BODY SHAPE DIVERGENCE AMONG WILD AND EXPERIMENTAL POPULATIONS OF WHITE SANDS PUPFISH (CYPRINODON TULAROSA)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Reports of contemporary evolution have become ubiquitous, but replicated studies of phenotypic divergence for wild populations are exceptionally rare. In 2001, a series of experimental populations were established to replicate a historic translocation event that led to a case of contemporary body shape evolution in the White Sands pupfish. Using landmark-based geometric morphometric techniques I examined phenotypic variation for seven of these populations, and two wild populations over a 5 year period (5-10 generations) in the field. Significant body shape divergence was observed, but divergence patterns were not parallel, suggesting that the ponds were ecologically dissimilar. Considerable body shape variation found among populations suggests that the observed divergence maybe governed by temporal environmental variance. In this study, body shape variation was correlated with population density. These data suggest that habitat intrinsic factors or unmeasured habitat features may have strong affects on body shape, warranting continuous monitoring of recently translocated fishes.

ACKNOWLEDGEMENTS

This study could not be possible without the collaborative efforts and support of many people, including: Dr. Craig Stockwell (North Dakota State University, Fargo, ND), Dr. Michael Collyer (Stephen F. Austin State University, Nacogdoches, TX), my committee members (Dr. Mark Clark, Dr. Steve Travers, and Dr. Marion Harris), Dr. Dean Adams (Iowa State University, Ames, IA), supporting graduate students and former experiment technicians, family, and friends.

Resources for this disquisition were provided by North Dakota State University, my major adviser, Dr. C. Stockwell, and collaborator, Dr. M. Collyer. Thanks to C. Stockwell for offering me this special opportunity and for encouraging my involvement with this long term project. Dr. C. Stockwell pursued the establishment of the experimental ponds to evaluate the evolutionary implications of altered selection pressures on White Sands pupfish. Experimental ponds were maintained with assistance from many people including C. Stockwell, M. Collyer, J. Terfehr, H. Reiser, D. Rogowski, D. Harstad, D. Moen, J. Vinje, and C. Teske. I am grateful for R. Myers (Range Geologist, Environmental Stewardship Division) and T.A. Ladd (Director, Environment and Safety Directorate, U.S. Army, White Sands Missile Range) in allowing arrangements for past range visitations. Thanks also to J. Pittenger, H. Reiser, C. Springer, and L. Dye for their assistance in obtaining permits and access to Holloman AFB. A special thanks to M. Collyer for his training on the fundamentals of geometric morphometric techniques, offering archived images from former White Sands pupfish collections, and assisting with multivariate data analysis. I appreciate all the comments and guidance on multiple drafts by my adviser, C. Stockwell, and committee members: Mark Clark, Steve Travers, and Marion Harris. Their

insightful comments greatly improved the quality of this thesis. Lastly, I thank Dr. Dean Adams for his insights on conducting a phenotypic trajectory analysis (side project) and in calculating Haldane rates. To all of these people I am especially grateful.

Financial support for the inception of this project was initially provided by the Department of Defense Legacy Resource Grant to Dr. Stockwell (Grant # DACA87-00-H-0014), which was administered by Dr. M. Hildegard Reiser, 49 CES/CEV, Environmental Flight, Holloman Air Force Base, NM. Collaborator, H. Reiser was central in obtaining funds to create the experimental ponds and for providing logistical support and field assistance. For this I am indebted. Additional funding for this project was provided by the Biological Sciences Department and Environmental Conservation and Sciences Program (ECS) of North Dakota State University, the National Science Foundation's East Asian and Pacific Summer Institute Program (EAPSI; Stipend Funds), C. Stockwell's indirect funds, and my own personal funds. Travel funds for oral and poster presentations were granted by the Environmental and Conservation Sciences Program, the North Dakota State University Graduate School, and the Biological Sciences Department. Tuition was paid, in full, by a Presidential Doctoral Fellowship award. I want to personally thank those involved in awarding this prestigious award, including the suggestive help from Dr. Meena Balgopal and Dr. Paul Ode in preparing for the application of this award, and Dr. David Wittrock (Dean; NDSU Graduate School), for his understanding and support to continue this funding for a Masters Project. Overall, I owe a debt of gratitude for the generosity and support of these people and programs.

Few students get the opportunities that I have had during my time at North Dakota State University, and I am grateful for this. A few highlights are provided below. From

the 12-16 November 2008, I attended the 40th Annual Meeting of the Desert Fishes Council in Cuatro Ciénegas, Coahuila, Mexico. The Cuatro Ciénegas Valley is comparable to the Galapagos Islands in terms of the amount of endemism. Craig Stockwell, S. Henkaneththegedara, J. Fisher, and I attended the conference and participated in multiple field trips in the valley - visiting the local ciénegas (springs) where we had a close look at some endangered fish species, such as the Cuatro ciénegas pupfish (*Cyprinodon bifasciatus)* and Cuatro ciénegas cichlid (*Herichthys minckleyi*). I extend a special thanks to C. Stockwell for financially facilitating this opportunity. From 1 March to 13 May 2009, I assisted a fellow graduate student, Sujan Henkanaththegedara, in evaluating the impacts of non-native western mosquitofish (*Gambusia affinis*) on endangered Mohave tui chub (*Siphateles bicolor mohavensis*) in Lake Tuendae, Mojave National Preserve, CA. Along with Justin Fisher, another graduate student in our lab, we were responsible for maintaining and conducting mesocosm experiments, building fish traps, and running mark and recapture surveys. Thanks to Robert Fulton, Jason Wallace, and Marcelo Aguirre for their company and generosity during our stay at the Desert Studies Center, Zzyzx, CA. From 16 June to 14 August 2009 I traveled to Wellington, New Zealand to study invasion dynamics of western mosquitofish. My New Zealand studies were awarded through an East Asian Summer Institute Program provided by the National Science Foundation. Here I collected specimens throughout the North Island of New Zealand and learned basic genetic techniques. These experiences could not have been possible without the collective support and collaboration from Dr. C. Stockwell, Dr. Peter Ritchie (Senior Lecturer), and Dr. Sébastien Rioux Paquette (post-doc) of Victoria University of Wellington, NZ. Lastly, from 21 February to 7 March 2010, I traveled to Nacogdoches, TX to study under Dr. M.

Collyer on geometric morphometric techniques. Few people have been as patient as M. Collyer, and I offer many thanks for offering his time. Collectively, without North Dakota State University none of these experiences would have been possible. Thanks for the wonderful journey.

I want to thank and acknowledge Dr. M. Balgopal and Dr. P. Ode for their help and encouragement for jumpstarting my graduate career. Without their initial support, my quest for higher education may not have been as successful. I am also thankful for my fellow colleagues in the lab, including: Steven Burdick (undergraduate volunteer; NDSU) S. Henkanaththegedara, Justin Fisher, Dave Mushet, Shawn Goodchild, and post-doc, Kevin Purcell. The presence, support, and consultations from these individuals were much appreciated.

Graduate school could not have been completed without the collective support of my family and friends. I would like to thank my lovely wife, Vanessa, my daughter, Alivia, my family, and my friends for their support in this endeavor. During my time at North Dakota State University, my wife and I were blessed with the birth of a beautiful baby girl, Alivia Marie Kowalski. Her presence positively contributed to the overall impetus in getting this disquisition finished. I would also like to personally thank my parents (including in-laws) for offering their homes for weekends and my time with the MN Department of Natural Resources based out of Fergus Falls, MN. For most of this period, my sister, Sarah, provided childcare services. I am indebted as her assistance played a critical role in helping me finish this thesis. To these people I give my most sincere thanks.

DEDICATION

This Thesis is dedicated to my wife, Vanessa and our daughter, Alivia.

PREFACE

Experimental History

The White Sands pupfish (*Cyprinodon tularosa*) is an imperiled desert fish species, classified as Threatened by the State of New Mexico, USA, because it is endemic to the endorheic Tularosa Basin, New Mexico. Native populations occur at Salt Creek (a saline river) and Malpais Spring (a brackish spring). New populations were established at Lost River in 1970 (a saline river) and at Mound Spring (a brackish spring; Pittenger and Springer 1999) sometime between 1967 and 1973. Both introduced populations were found to be genetically descended from Salt Creek (Stockwell and Mulvey 1998). Collyer et al. (2005) reported significant morphological divergence among these four populations, based on geographically divergent selection. Overall body depth was significantly more pronounced in both Malpais Spring and Mound Spring than Lost River and Salt Creek. Notably, these patterns were found to be consistent with the abiotic conditions. Fish from both brackish spring environments were characterized as having deeper body shapes than fish from both saline river environments. These observed departures in body shapes were attributed as an adaptive response to both environmental salinity and flow (Collyer 2003; Collyer et al. 2005).

In 2001, fifteen experimental "refuge" populations with conditions similar to Mound Spring were established within artificially created earthen experimental ponds to 'replicate' the historic Mound Spring translocation event. Among the 15 artificial ponds, 9 received fish from Salt Creek and 6 received fish from Lost River. However, before the end of September 2001, five experimental ponds were lost in a flood event, and a 6th pond extirpated within the first two years (see Table D.1 in Appendices). By 2006, 7 of the 9

original Salt Creek derived experimental populations survived and are the focus of this disquisition.

The establishment of experimental populations allowed for the experimental evaluation of altered selection on White Sands pupfish in the wild. In a previous study, Collyer et al. (2007) examined body shape variation among these populations after one year to determine if the predicted morphological divergence toward a deeper body shape had occurred. After one year, body shape divergence among experimental ponds was found to be sufficiently smaller than the observed 30 year old divergence reported for Mound Spring. However, experimental pond shapes were found to be intermediate between Salt Creek and Mound Spring and in the direction of the predicted divergence, suggesting that phenotypic plasticity was facilitating the directional evolution of body shape deepening (Collyer et al. 2007).

Prior to the termination of this experiment, in 2006 an additional set of samples were collected from Salt Creek, Mound Spring, and experimental pond populations, lending the opportunity to quantify the amount of morphological change over a 5 year period (5-10 generations).

Thesis Composition

This thesis is organized by two chapters, which are supported by several appendices. The first chapter is a general introduction to 1) desert aquatic ecosystems, 2) conservation management, 3) pupfishes, and 4) White Sands pupfish (*Cyprinodon tularosa*). The second chapter is an investigation into the body shape divergence among wild and experimental populations of the White Sands pupfish over a 5 year period. This chapter has multiple authors; coauthors are included within the manuscript title. Chapter 2

was prepared and formatted for submission to *Ecological Research*. As such, detailed material unnecessary for the publication of Chapter 2 was moved to the appendices (see below for organization). For the work reported, I was both the primary author and person responsible for the data analyses and interpretation of the results. I was however not the person who designed and initiated the project. In 2001, Dr. Craig A. Stockwell, with the efforts of others (see experimental history section above), conceived and designed the experimental approaches for the evaluation of contemporary body shape evolution among White Sands pupfish in the wild. As a result, a large component of this thesis is supported and constrained by former work conducted by Dr. Craig A. Stockwell, Dr. Michael L. Collyer, and others (see Acknowledgements).

A detailed description of the methods used for the quantification of body shape is provided in the appendices. Ancillary materials supporting Chapter 2 are also provided. Appendices are formatted so that all information is organized by precedence, and includes 1) a detailed description of the methods and statistical analyses, 2) a summary of the complete White Sands pupfish collection, 3) summary data for founding experimental pupfish, 4) the fate of the experimental ponds, 5) the repeatability (i.e. measurement error) and placement of the thirteen landmark configurations used for quantifying body shape, 6) allometric relationships for the completed White Sands pupfish collection, 7) the computed probabilities of random distances generated in Chapter 2, 8) calculated rates of divergence (i.e., Haldanes) among experimental White Sands pupfish populations, 9) additional laboratory experiments exploring the suitability of a surrogate pupfish species, artifacts of preservation within geometric morphometric studies, and evidence of functional body shape advantages to water flow, 10) a primer on geometric morphometrics, and 11) a brief

glossary of geomorphometric terms and symbols to avoid any ambiguity or confusion among concepts. References are provided at the end of each chapter and at the very end, covering the entire thesis.

Animal Welfare and Experimental Approval

The former collections and research conducted for this work involved vertebrate animals. This Research was approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC). White Sands pupfish (*Cyprinodon tularosa*) were collected under the IACUC Protocol #A0117 from the White Sands Missile Range. Collections were made under the New Mexico State collecting permit 2887 permitted to Craig A. Stockwell. Sheepshead minnows (*Cyprinodon variegatus*) were obtained from a commercial supplier; Aquatic Biosystems Inc. located in Ft. Collins, Colorado, U.S.A. Experimental use of Sheepshead minnows was approved by the North Dakota State University IACUC Protocol # A0902. This thesis was approved by Operation Security (OPSEC) for public release by the White Sands Missile Range, distribution unlimited; OPSEC review was completed on 03 February, 2012.

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CHAPTER 1. INTRODUCTION

Brandon M. Kowalski [1]

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Desert Aquatic Ecosystems

Severe conditions exist among desert aquatic ecosystems within the southwestern United States. In effect, aquatic systems are usually rare, widely dispersed, and generally characterized by relatively simple, isolated communities. Unlike most complex communities, these small, simple habitats can be artificially replicated and experimentally controlled under natural conditions, making them ideal for empirical studies (Naiman and Soltz 1981; McMahon and Tash 1988).

Fishes that have managed to persist among many desert aquatic environments provide a unique opportunity to study adaptive characteristics. The study of such divergence is facilitated by the fact that many desert fishes can be easily experimentally manipulated because most are relatively small and can be easily bred and reared (Johnson and Jensen 1991; Stockwell and Leberg 2002).

Present day fish distributions throughout the south western United States are directly related to the geologic history within the Pleistocene epoch (≥ 10,000 years ago; Miller 1981; Smith 1981). Nearly all enclosed basins historically held water, varying in both size and duration. In fact, almost all present day aquatic environments were inundated by large Pleistocene lakes (Miller 1948). However, as conditions changed, the lakes desiccated, and today only small remnant water bodies remain. Evidence for the existence of these great lakes is presented in the remnant fish fossils (Spencer et al. 2008), as well as beach terraces visible high on adjacent hills.

Today these remnant aquatic habitats include small, isolated ponds, pools, springs and rivers (Brown 1971, Smith 1981), which are characterized by wide environmental variation in temperature, salinity, flow, and constancy. Such high variance from extreme conditions can drive diversification of fish traits associated with local adaptation, such as physiology, behavior, morphology, and life history (Miller 1948). Consequently, the isolation of aquatic organisms has resulted in rapid diversification of fishes over a relatively short temporal scale (Miller 1948). Further, desert fishes have been exclusively translocated to novel habitats, providing the opportunity to study phenotypic divergence on contemporary time scales (up to a few hundred generations; Stockwell and Leberg 2002; Wilcox and Martin 2006).

Conservation of Icthyofauna in Desert Ecosystems

Currently, aquatic habitats within desert ecosystems are at serious risk. The physical alteration of aquatic habitats has been noted as the primary factor for declines in native fishes (Minckley and Deacon 1968; Miller 1972; Miller et al. 1989). Anthropogenic factors such as overgrazing by domesticated livestock, ground water mining, fire suppression, introduction of non-native species, and climate change, are only a few examples that have contributed to unfavorable conditions for desert fishes (Minckley and Deacon 1968; Williams et al. 1985; Grover and Musick 1990; Hendrickson and Brooks 1991; Fagan et al. 2002). These impacts have taken a notable toll on biodiversity (Dudgeon et al. 2006; Vörösmarty et al. 2010). According to Pister (1974), within 34 years, 4 species and 6 subspecies from 6 genera appeared to have gone extinct, with an additional 50 taxi becoming threatened. By 1989, Miller et al. (1989) reported confirmed

extinctions among 3 genera, 27 species and 13 subspecies of North American fishes (within the last 100 years).

Not surprisingly, population introductions have become a common method for the conservation (Soule 1985) of threatened and endangered fish species in the Southwestern United States (Williams et al. 1988; Minckley 1995). This includes the translocation of managed fishes from their current habitat to additional natural localities or propagation facilities (Minckley 1995; Stockwell and Leberg 2002). For imperiled species, translocations primarily function to address the vulnerability of a species by improving its security from catastrophic losses. Interestingly, more than 80% of recovery plans of endangered or threatened fishes call for some type of translocation (Williams et al. 1988).

Early introduction efforts were often carried out opportunistically with minimum planning (Hendrickson and Brooks 1991). As a consequence, many attempts failed because recently established populations were often genetically compromised via founder effects, bottlenecking, and genetic drift (Stockwell et al. 1996). As such, the establishment of populations in novel environments may often be associated with rapid evolutionary divergence (Stockwell and Weeks 1999; Reznick and Ghalambor 2001).

Ineffective or even detrimental management practices may result when microevolution is not considered (Ashley et al. 2003). For instance, inappropriately managed translocated populations may rapidly diverge from their parental population and become maladapted to their ancestral habitat (Frankham et al. 1986; Stockwell et al. 2003). Documentation of rapid adaptation is quite common across a variety of species (reviewed in Reznick and Ghalambor 2001, and Stockwell et al. 2003). Evolutionary divergence of such recently established populations is likely to diminish their conservation value

(Stockwell and Weeks 1999; Wilcox and Martin 2006). Thus it is important to monitor these translocated populations (Ashley et al. 2003; see below).

Ecology and Evolutionary Biology of Pupfishes

Pupfishes provide a good model system for assessing the effects of translocation because many pupfish are threatened or endangered (see Williams et al. 1985), and their conservation plans support or contain a component of replication (i.e., $\geq$ 12 species; Deacon and Deacon-Williams 1991; Hendrickson and Brooks 1991). Pupfish are a small group of killifish belonging to 10 genera representing over 100 species within the Cyprinodontidae family (Moyle 2002). Approximately 20 species of *Cyprinodon* occur in aquatic habitats throughout the southwestern deserts of United States and Mexico, often living in full geographic isolation from each other (Turner and Liu 1977; Moyle and Cech 2000). Pupfishes are thus similar to the Darwin Finches of the Galapagos Islands (Boag and Grant 1981) in that they offer an opportunity to observe the process of evolution in real time.

Species in this family are typically noted for occupying a wide range of habitats, with salinities ranging from brackish environments to four times seawater (i.e., 4 x 35 ppt), temperatures from freezing to 45°C, and oxygen levels from normal to hypoxic levels of 1.5 mg/L (Miller 1948; Barlow 1958; Martin 1968; Miller1981; Bennett and Beitinger 1997; Beitinger et al. 2000; Rogowski et al. 2006). Due to these extreme conditions, most desert communities exclude intolerant species, making them simple systems for study (Bayly 1972; Herbst 2001; Rogowski 2004).

"Cyprino-don," translated, means carp with teeth (Moyle 2002). These fish received their name due to their minnow-like appearance and single series of incisor-like

tricuspid teeth on the lower and upper jaws. Pupfishes are typically small (i.e., ranging from 2.5-7.5 cm depending on species) and tend to live no longer than 2 to 3 years (Moyle 2002). Pupfish are characterized by large eyes, deep-rounded bodies, posteriorly positioned dorsal and anal fins, rounded caudal fins, nuchal/ dorsal crests, and small terminal protrusive lower jaws (Figure 1.1), all suggesting that pupfish have adapted to a standing, slow-moving mid-water lifestyle (Constantz, 1981).

Adult pupfish are usually sexually dimorphic, where males are more elaborately colored than females (Moyle 2002). Male pupfish tend to be aggressive and display lek-like behavior (Loiselle and Barlow 1978). Interestingly, the name "pupfish" was coined by Carl L. Hubbs for their observed playful behavior, derived from the lekking behavior

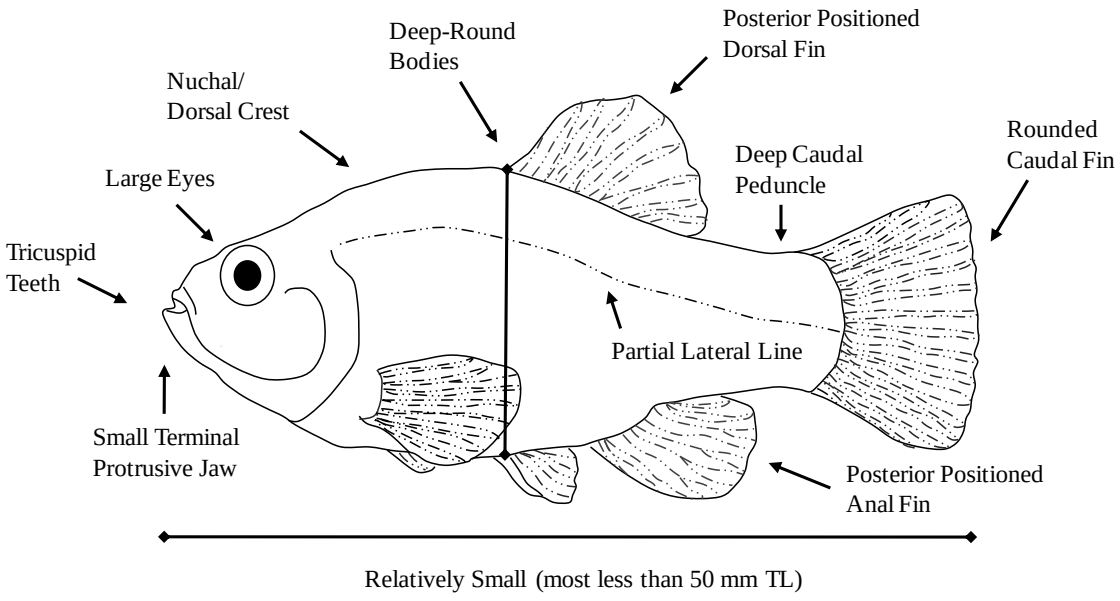

Figure 1.1 General characteristics of a pupfish (Family: Cyprinodontidae). Details generated from Moyle (2002). Illustration by Brandon M. Kowalski.

demonstrated by aggressive males (Kodric-Brown 1978; Moyle 2002). Gravid females visit shallow littoral 'lekking' grounds in search for mates. Depending on pupfish density (Kodric-Brown 1986), males use one of the following breeding strategies to court available females: 1) territorial, 2) floater or satellite, and 3) sneaker (Barlow 1961; Kodric-Brown 1986; Watters et al. 2003), however see Kodric-Brown (1981) on group spawners. After a brief courtship, females will lay a single egg and adhere it to substrate within the male's territory (Kodric-Brown 1977). Gestation periods generally are between 4-10 days (Moyle 2002). Pupfishes lack direct parental care and are generally known to cannibalize eggs and young, especially in high density populations (Liu 1969; Soltz 1974).

Morphological and life history variation among pupfish populations is generally variable depending on the environment. Pupfish inhabiting springs typically exhibit smaller, chubbier body shapes, lower fecundity, greater parental investment (e.g., egg size and territorial defense), and longer breeding seasons than pupfish from stream environments (Constantz 1981). Moreover, it has been generally established in systematic studies of fishes that meristic characteristics tend to increase in number with increasing salinity (Miller 1948; Miller and Echelle 1975).

Ecology of White Sands Pupfish in the Tularosa Basin, New Mexico

Formally described by Miller and Echelle in 1975, the White Sands pupfish (*Cyprinodon tularosa*), Family Cyprinodontidae, is a member of the sheepshead minnow (*Cyprinodon variegatus*) complex (Miller and Echelle 1975; Echelle et al. 2005). White Sands pupfish have deep bodies that are laterally compressed, giving them a relatively robust look (Figure 1.2). White Sands pupfish rarely exceed 50 mm in standard length, but have been documented at 56 mm (Miller and Echelle 1975; Jester and Suminski 1982).

Adult individuals exhibit sexual dimorphism (Figure 1.2) (Miller and Echelle 1975; Stockwell 2002; Collyer et al. 2005). Males are deep bodied and display prominent dorsal/nuchal crests with prominent metallic blue coloration along their upper dorsal region, grayish blue flanks, yellow-orange coloration along paired fins and distal margins of their anal and dorsal fins, and a distinct black terminal band on the caudal fin (Figure 1.2). Females are characterized by a whitish to silvery hue, displaying grayish to golden striated bars along their flanks. Upper dorsal regions are olivaceous in color and dorsal fins display pigmentation (i.e., ocellus) among posterior membranes (Figure 1.2).

Reproductively, White Sands pupfish mature and breed readily within about 1-2 years, reaching a maximum longevity of ~5 years (Jester and Suminski 1982); however, fish rarely exceed 24 months (Propst 1999). Spawning typically begins in early spring when waters reach temperatures of 18°C, but may extend well into the fall (Suminski 1977). Males establish and guard territories (leks) in shallow vegetated littoral zones of springs and in low-flow, vegetated margins of streams (Suminski 1977; Propst 1999). Males engage in ritualized movements to attract females. Females lay single eggs and adhere them to substrate (Jester and Suminski 1982), while the male wraps his anal fin around the female's vent (e.g., see Barlow 1961; Propst 1999). Females typically spawn twice within a 24 hour period (Propst 1999). Total fecundity may range on average from 810 to 2,900 ova per female throughout the spawning season. Eggs typically hatch within about a week (Jester and Suminski 1982).

The White Sands pupfish is omnivorous, feeding on organic detritus, algae, diatoms, and other aquatic invertebrates (Propst 1999; Pittenger 2010); however, mosquito larvae are believed to be a food of choice (Suminski 1977). Few predators of White Sands

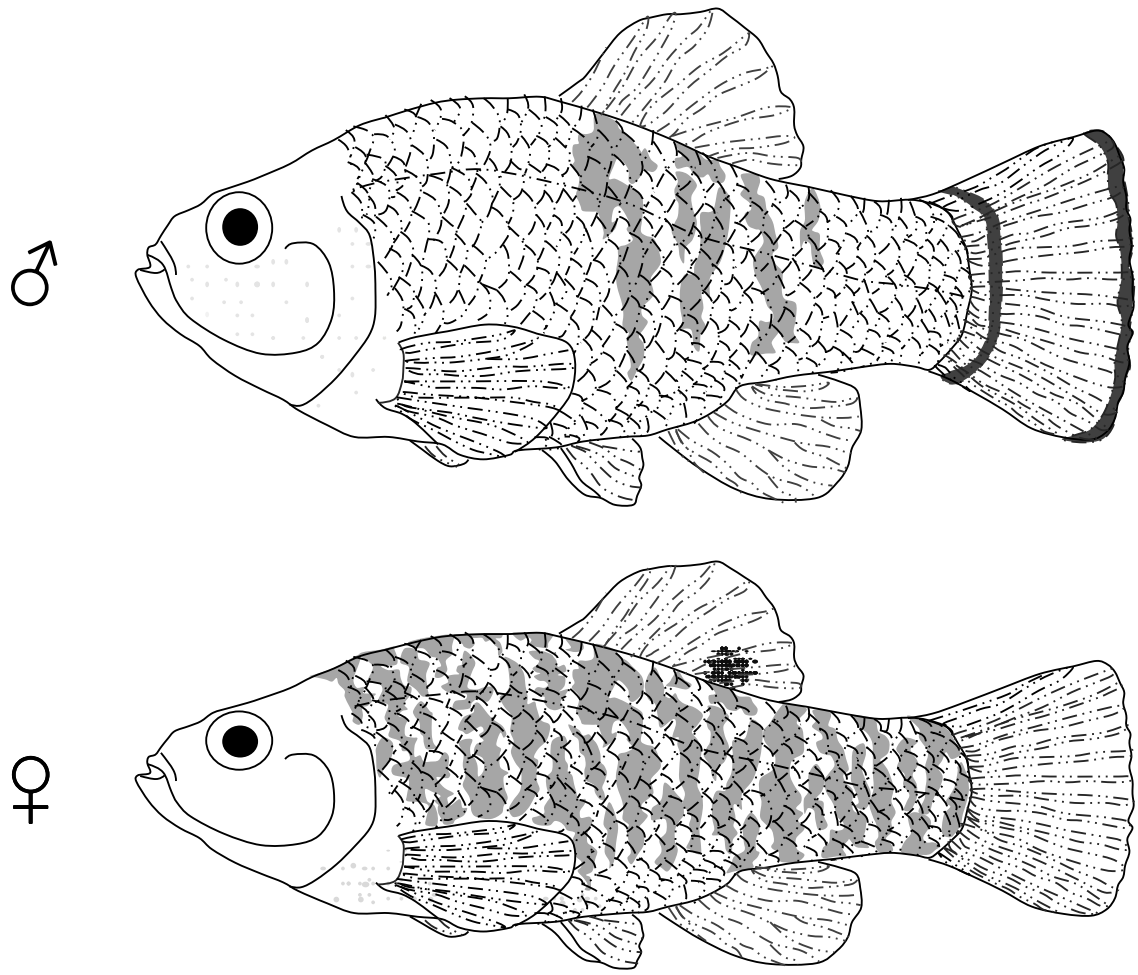

Figure 1.2 Illustration of adult male (top) and female (bottom) White Sands pupfish (*Cyprinodon tularosa*). Illustrations by Brandon M. Kowalski.

pupfish exist, although dragon fly larvae (Order: Odonata), dytiscids, belostomatids, and herons (Family: Ardeidae; e.g., great blue heron (*Ardea Herodias)*) are present at some White Sands pupfish sites (Rogowski et al. 2006; C A Stockwell, Pers. Comm.). Notably, parasites (White Grubs, Family: Diplostomatidae; *Gyrodactylus tularosae* Kristy and Stockwell; Heterophydae; *Ascocoytl*) have also been shown to occur among some sites (e.g., see Collyer and Stockwell 2004; Kritsky and Stockwell 2005; Moen and Stockwell 2006; Rogowski and Stockwell 2006; Stockwell et al. 2011).

Like most pupfishes in the *Cyprinodon* genera, White Sands pupfish tolerate a wide range of salinities and temperatures (Stockwell and Mulvey 1998, Rogowski 2004, Rogowski et al. 2006). The White Sands pupfish can be found in salinities ranging from brackish (1.5 ppt) to salinities three times that of sea water (>75.2 ppt) and temperatures ranging from cool (3.2 °C) to hot (35.9 °C) depending on the aquatic system (see Table 2.1; Collyer 2003; Rogowski 2004; Rogowski et al. 2006).

The history of the White Sands pupfish is closely linked to the geological history of the endorheic Tularosa Basin located between the Sacramento and San Andres mountains of south central New Mexico (Figure 1.3; Herrick 1900; Miller and Echelle 1975; Pittenger & Springer 1999). The White Sands pupfish is the only endemic fish in this basin and has been geographically isolated since the Pleistocene (Miller and Echelle 1975). Historically, this region was inundated within the basin of the Pleistocene Lake Otero, reaching a maximum depth of 1,250 m approximately 5,000 to 7,000 years ago (Herrick 1904; Pittenger and Springer 1999). As the climate warmed, conditions became drier, and the lake receded, eventually desiccating to the current conditions.

The formation of the Malpais lava flow (also known as the Carrizozo lava flow) from the eruption of little Black Peak roughly 5,000 years ago (Dunbar 1999) contributed to the alteration of the known major surface drainage patterns in the Tularosa Basin today. The Malpais lava flow seen from the aerial photo of the region in Figure 1.3 ultimately resulted in the fragmentation of the largest remnant tributary (i.e., proto Salt Creek tributary) and the formation of a novel artesian spring (Malpais Spring). Overall, ten remnant aquatic habitats within the White Sands pupfish natural range resulted from the combined desiccation of Lake Otero and the formation of the Malpais lava flow (Jester and

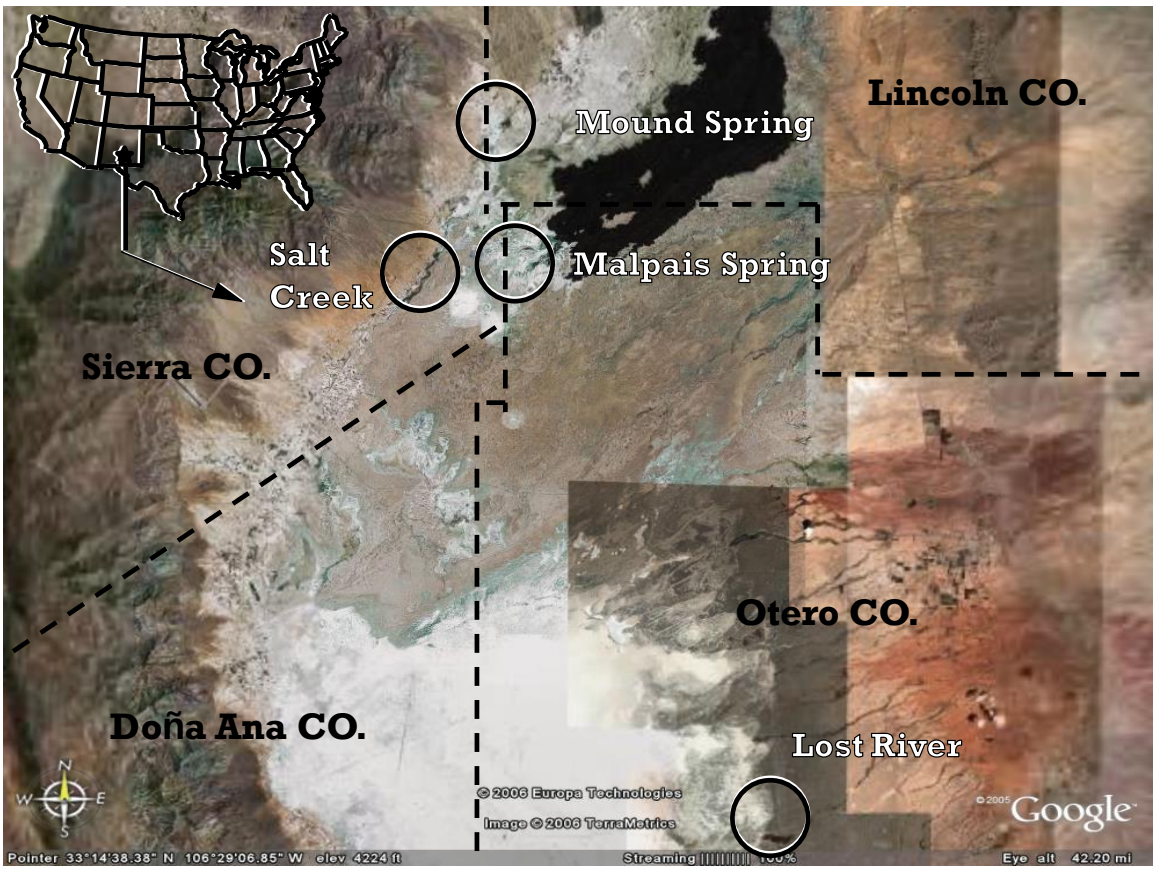

Figure 1.3 Aerial photo of the Tularosa Basin, NM depicting four wild White Sands pupfish habitats (encircled). The Malpais lava flow is depicted in black. Both Salt Creek and Lost River are lotic and highly saline; whereas, Malpais Spring and Mound Spring are lentic and have brackish characteristics. Mound Spring and Lost River were founded by Salt Creek in the 1970's. Image obtained from Google Earth ©.

Suminski 1982; Pittenger and Springer 1999). These habitats are now sustained by either surface water runoff, ground water, or a combination the two.

Today, the Tularosa Basin is typically described as an arid, hot, and windy environment. The White Sands pupfish has persisted, but is classified as a threatened species within the state of New Mexico (19 NMAC 33.1) and federally listed (50 CFR 17:64481-64485) as a species of concern (Miller and Echelle, 1975). White Sands pupfish currently occupy four naturally isolated sites within the Tularosa Basin including, Salt

Creek in Sierra County, Mound Spring in Lincoln County, and Malpais Spring and Lost River in Otero County (Figure 1.3 and Figure 1.4). Notably, Salt Creek, Mound Spring, and Malpais Spring are located within the White Sands Missile Range (WSMR), and Lost River is located within the Holloman Air Force Base (HAFB). Additional populations were established in 2006 at Camera Pad Road Pond, Weenis (Salt) Spring, and two ponds on a golf course, all on the Holloman AFB (C A Stockwell, Pers. Comm.). Recent surveys suggest that both golf course populations did not establish (Jill Wick, New Mexico Department of Game & Fish, Pers. Comm.). These fish are all Salt Creek descendants that were translocated from a series of experimental ponds (see Chapter 2) by Dr. Craig A. Stockwell (North Dakota State University) on 18-21 December 2006. More detailed information on the physical characteristics of these habitats is found elsewhere (e.g., see Pittenger and Springer 1999; Collyer 2003; Rogowski 2004; Rogowski et al. 2006; Pittenger 2010).

Using hydrogeographic and historic information, Pittenger and Springer (1999) concluded that Salt Creek and Malpais Springs were the only historic native habitats for White Sands pupfish, presumably isolated since the desiccation of Lake Otero (i.e., the Pleistocene Epoch roughly 10,000 years ago). Populations of White Sands pupfish were established in the 1970's at Lost River (i.e., in 1970 for mosquito control) and sometime between 1967 and 1973 at Mound Springs (i.e., for unknown reasons; Pittenger and Springer 1999). Genetic studies conducted by Stockwell et al. (1998) showed both Lost River and Mound Spring were genetically descended from Salt Creek. They also recognized Malpais Spring and Salt Creek populations as two evolutionary significant units

Figure 1.4 Depiction of the four natural White Sands pupfish habitats: A) Malpais Spring, B) Salt Creek, C) Mound Spring, and D) Lost River. Both Salt Creek and Lost River are lotic and highly saline; whereas, Malpais Spring and Mound Spring are lentic and have brackish characteristics. Images acquired from archived White Sands Missile Range photos (B & C) and Craig A. Stockwell (A & D).

(ESU) for being genetically and ecologically dissimilar, suggesting that any future management should conserve both ESUs.

White Sands pupfish habitats differ considerably from each other in terms of temperature, salinity, and water flow. Salt Creek and Lost River are both lotic environments and highly saline; whereas, Malpais Spring and Mound Spring are lentic and brackish in nature (Figure 1.4; Table 2.1; Miller and Echelle 1975; Pittenger and Springer 1999; Pittenger 2010). Detailed morphometric and meristic data suggest that White Sands

pupfish display an adaptive ecological association of body shape to their corresponding environment. White Sands pupfish from more saline environments characteristically are more slender bodied than fish from more brackish environments (Miller and Echelle 1975). Collyer et al. (2005, 2007) demonstrated strong correlative effects of salinity on morphology of the White Sands pupfish. In general, White Sands pupfish from more saline environments were more streamlined with smaller and more numerous scales than those observed from brackish environments (Miller and Echelle 1975; Collyer et al. 2005, 2007). Because body shape has a functional aspect (Miller 1948) and reflects phenotypic integration (Bookstein 1991), these associations were viewed as a population response to local selection. Additional evidence of a genetic basis for these observed body shape differences was later documented (Collyer 2003, Collyer et al. 2011).

Experimental evidence supporting predictable body shape patterns under different environments may have important adaptive applications for the conservation of fishes. In the case of White Sands pupfish, the species recovery plan calls for the establishment of additional refuge populations. Under these conditions, newly established populations may rapidly diverge from their parental population and become maladapted to their ancestral habitat (Frankham et al. 1986; Lande and Shannon 1996; Ford 2002; Stockwell et al. 2003; Kinnison et al. 2007; Collyer et al. 2011). Because refuge populations should unequivocally represent genetic replicates of native populations (Vrijenhoek et al. 1985; Echelle 1991; Stockwell et al. 1996, 1998), the evolutionary consequences of moving adapted populations to novel environments was explored in Chapter 2.

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CHAPTER 2. BODY SHAPE DIVERGENCE AMONG NATURAL AND EXPERIMENTAL POPULATIONS OF THE PROTECTED WHITE SANDS PUPFISH (CYPRINODON TULAROSA)

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Abstract

The creation of refuge populations is a commonly used tool for the conservation of protected species, yet studies of these events are hampered by a lack of replication. In 2001, a series of experimental ponds were established to replicate a historic translocation event of the protected White Sands pupfish (*Cyprinodon tularosa*). Previous morphological studies showed that phenotypic divergence of the Mound Spring population of *C. tularosa* from the ancestral Salt Creek population had occurred over a 30 year period. The introduction of pupfish to Mound Spring resulted in significant departures in both body shape and allometry. In this study we used landmark-based geometric morphometric techniques to test if body shape divergence could be experimentally replicated among a series of experimental pond populations monitored over a 5 year period (5-10 generations) in the field. Our study design allowed us to evaluate body shape divergence of ancestral (allochronic) and derived (synchronic) populations. Based on allochronic comparisons alone, we found little evidence of significant body shape divergence; however additional work suggested that these comparisons are limited due to inherent preservation effects.

Conversely, almost all synchronic comparisons, which were not confounded by preservation effects, were highly significant. Considerable body shape variation for both wild and experimental populations suggests that the observed divergence maybe governed by temporal environmental variance. We found evidence that is suggestive of density-dependent effects on body shape. Despite concerted efforts to replicate the Mound Spring habitat, these data suggest that habitat intrinsic factors or unmeasured habitat features may have strong effects on body shape, illustrating the need to monitor phenotypic divergence when managing rare species.

Introduction

To date, translocations have been an important strategy for the conservation of imperiled fish species (Williams et al. 1988; Schooley and Marsh 2007). Most current recovery plans of threatened or endangered fish species contain a component for the translocation of individuals to new locations to increase the probability of persistence (Griffith et al. 1989; Minckley 1995; Minckley et al. 2003). However, this form of bet-hedging against potential extirpation or extinctions may result in unplanned biological consequences (Hendrickson and Brooks 1991, Stockwell and Weeks 1999; Stockwell and Leberg 2002).

Conservation practices such as captive breeding and the creation of refuge populations (e.g. see Baugh and Deacon 1988; Winemitter and Anderson 1997) may facilitate unintentional phenotypic divergence (Frankham et al. 1986; 2008; Fleming and Einum 1997; Stockwell and Weeks 1999). For example, original selection pressures may be relaxed and new ones imposed on organisms in novel conditions, resulting in divergence at the population level (Stockwell et al. 2006). Under these conditions, newly established

populations may rapidly diverge from their parental population and become maladapted to their ancestral habitat (Frankham et al. 1986; Lande and Shannon 1996; Ford 2002; Stockwell et al. 2003; Kinnison et al. 2007; Collyer et al. 2011).

Divergence of introduced populations, which can occur in a few generations, may reflect phenotypic plasticity and/or contemporary evolution (Kinnison and Hendry 2001; Rice and Emery 2003; Stockwell et al. 2003). Experimentally, transplants have shown to be a valuable tool for comparative studies of phenotypic divergence. Such responses have been well documented throughout the literature, including for example, observed changes in morphological (Losos et al. 1997; Kinnison et al. 1998a; Carroll et al. 1998; Collyer et al. 2005, 2011; Wilcox and Martin 2006), and life history traits (Reznick et al. 1990; Scribner et al. 1992; Kinnison et al. 1998b; Stockwell and Weeks 1999; Stockwell and Vinyard 2000; Haugen and Vøllestad 2001; Kinnison et al. 2001; Quinn et al. 2001).

Desert fishes provide an opportunity to study phenotypic responses of introduced populations in the wild because 1) many conservation plans typically contain a component for the creation of refuge populations (Chapter 1; Deacon and Deacon-Williams 1991; Hendrickson and Brooks 1991; Stockwell and Weeks 1999; Stockwell and Leberg 2002; Collyer et al. 2007) and 2) introduction histories are typically known or can be reconstructed (Stockwell et al. 1996, 1998). Despite such utility, replication of transplant events in the field is rarely considered for experimental evaluation (Stockwell and Leberg 2002; Stockwell et al. 2003). Here, we examined morphological divergence of 7 experimental 'refuge' populations that were established to replicate a historic translocation event and determine if phenotypic divergence could be experimentally replicated in the field.

Study System

The White Sands pupfish (*Cyprinodon tularosa*; Miller and Echelle 1975) is an imperiled desert fish species located in the endorheic Tularosa Basin, New Mexico (Propst 1999; Stockwell 2002). Native populations of White Sands pupfish occur at Salt Creek and Malpais Spring; although, additional White Sands pupfish were introduced and established from Salt Creek (Stockwell et al. 1998) to Lost River in 1970 and to Mound Spring sometime between 1967 and 1973 (Figure 2.1) (Pittenger and Springer 1999). Mound Spring and Lost River are putative refuge populations for the Salt Creek population; however, these habitats are very different in terms of salinity, water flow, and temperature. Both Malpais Spring and Mound Spring are brackish springs with little appreciable flow or temperature variation; whereas, both Salt Creek and Lost River are highly saline with greater variation in both flow and temperature (Stockwell and Mulvey 1998).

Earlier morphological work on the White Sands pupfish indicated that the introduction of pupfish to Mound Spring resulted in significant departures in body shape. Mound Spring fish diverged from a streamlined Salt Creek body shape to a deeper body shape within a 30 year period (Collyer et al. 2005). These pronounced shape changes were consistent with the degree of abiotic change; where, body shape significantly correlated with differences in salinity. Additional experimental work suggested body shape to be heritable, and the divergence at Mound Spring to be a case of contemporary evolution (Collyer et al. 2011).

Methods

To determine if phenotypic divergence can be experimentally replicated, the Mound Spring translocation event was replicated by translocating fish from Salt Creek to a series

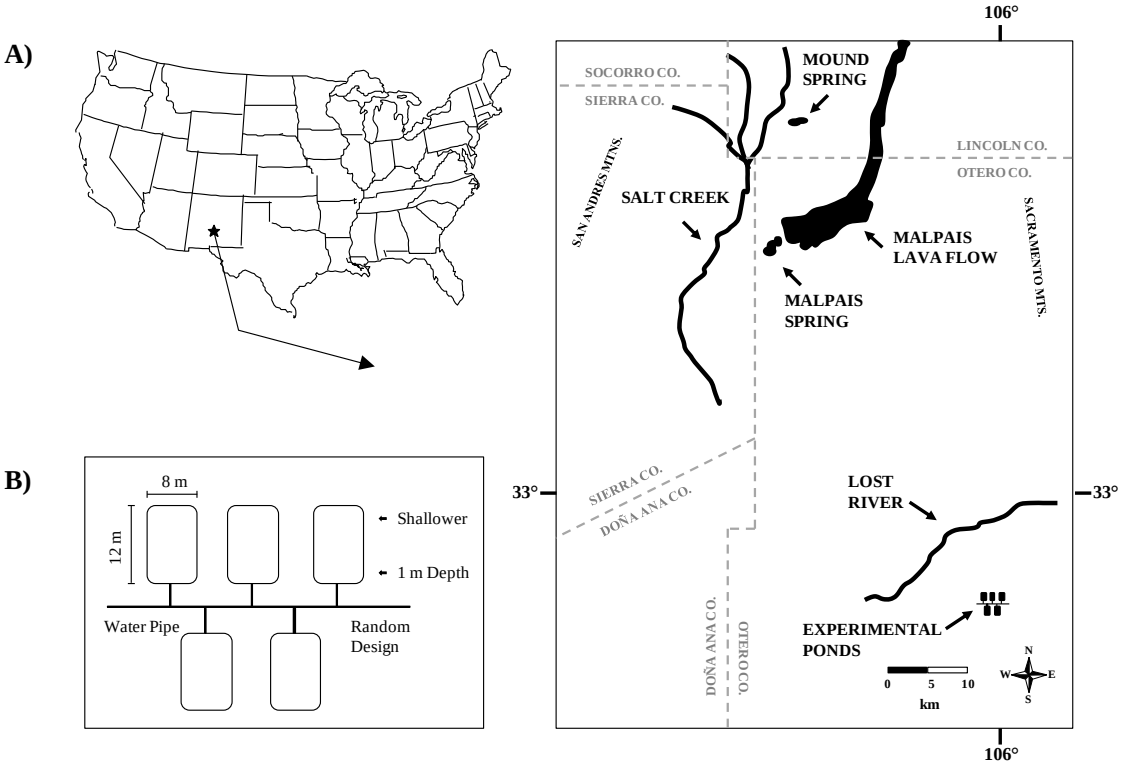

Figure 2.1 A) Geographic distribution of wild and experimental White Sands pupfish populations located within the Tularosa Basin, New Mexico (habitats exaggerated). Circa 1970, Salt Creek (SC) populations were introduced to two novel habitats, 1) Mound Spring (MO) and 2) Lost River (LR). In 2001, nine Salt Creek derived experimental populations of White Sands pupfish were established within artificial-earthen experimental ponds to replicate the Mound Spring translocation event. By 2006, 7 of the 9 experimental ponds persisted and are the focus of this study. B) Schematic of experimental ponds. Ponds were staggered with 8 x 12 m dimensions (only 5 ponds shown). Ponds were 1 m deep and gradually became shallower. Water was supplied by a main pipeline. Pond schematic modified from Collyer (2003; Pg. 86) with permission. Illustrations by Brandon M. Kowalski.

of experimental ponds, which mimicked the ecological conditions of Mound Spring (See Collyer et al. 2007). We examined 7 of these experimental populations, as well as the Salt Creek and Mound Spring populations, to evaluate whether phenotypic divergence in body shape is deterministic. Because body shape has a functional aspect (Miller 1948) and reflects phenotypic integration (Bookstein 1991), body shape divergence was viewed as a population effect to local selection and as a measure of biodiversity (Miller and Echelle

1975). Evidence supporting this hypothesis would suggest that selection within experimental ponds favors a common morphology. Furthermore, because experimental ponds were designed to be ecologically similar to Mound Spring, we predicted that shape changes should converge in a parallel (similar) direction toward a Mound-like body form (i.e., parallel shifts due to consistent responses to similar selection; see Losos 1998; Losos et al. 2001).

Experimental Pond History

In 2001, a series of experimental ponds were constructed on the Holloman Air Force Base, NM (Figure 2.1) (Collyer et al. 2007). All artificial ponds were constructed with an 8 x 12 m rectangular surface dimension and arranged in a staggered design along a water supply line (Figure 2.1B). Ponds were initially filled in March 2001 using tertiary treated sewage effluent (chlorine removed) from the Holloman Air Force Base water treatment plant and maintained by a spigot (positioned 1.5-2.0 m above the center of each pond) to a depth of approximately 1 m. Ponds were subsequently left to establish an aquatic invertebrate and macrophyte community prior to fish introductions. Breeding substrate was supplied (Collyer et al. 2007).

In late May to early June 2001, two-hundred White Sands pupfish (100 male and 100 female young-of-the-year) were introduced from Salt Creek to each of 9 experimental ponds (Table C.1). By August 2001, reproduction was observed in all experimental ponds (Collyer et al. 2007). White Sands pupfish mature and breed readily within 1 year and generally do not live more than 2 years (Propst 1999; Rogowski 2004). Thus, 5-10 generations occurred over the duration of this study.

In September 2001, two experimental ponds were lost in a flood event, and a third experimental pond (P-11) leaked into an unfilled pond due to subterranean erosion. However, because P-11 was not compromised, we kept this population in our analyses. By 2006, 7 of the 9 original Salt Creek derived experimental populations persisted (Table D.1) and are the focus of this study.

Ecological Characterization of Habitats

Physical characteristics, including temperature and salinity, were measured with a portable instrument (Yellow Springs Instrument-YSI 85®; Yellow Springs, OH) approximately weekly for each experimental pond since their inception in May 2001 until February 2006 (Table 2.1). During this same period, minimal records were kept for wild habitats due to security and logistical limitations. Therefore, data summarized in Stockwell and Mulvey (1998) and Rogowski (2004) for Salt Creek and Mound Spring (collected from 1996, 1997, 1999-2002) were combined and summarized in Table 2.1 for comparisons.

Relative similarities (or dissimilarities) among habitats were explored by performing a nonmetric multidimensional scaling procedure (NMDS; Manly 1994; *sensu* Collyer 2003 and Collyer et al. 2005). Measured characteristics used to perform the NMDS included average salinity and range, average temperature and range, and relative flow (1 = high water flow in at least portions of the habitat, 2 = some water flow, 3 = no water flow; Collyer 2003) to describe the ordered ecological relationships. NMDS were performed using 9999 randomly started ordinations.

Data Collection and Quantification of Body Shape

A total of 1120 White Sands pupfish specimens were obtained from 7 experimental and 2 wild populations over the course of a 5 year period (Table 2.2). Pupfish were

Table 2.1 Recorded habitat characteristics for experimental and wild populations of White Sands pupfish.

	Temperature (°C)			Salinity (ppt)			
Wild Habitats[†]	Min.	Mean	Max.	Min.	Mean	Max.	Rel. Flow[‡]
MO	8.7	6.1	28.8	1.5	3.0	4.0	3
SC	5.2	17.3	32.6	10.2	17.4	28.1	1
Experimental Ponds[*]	Min.	Mean	Max.	Min.	Mean	Max.	Rel. Flow[‡]
8	3.9	16.8	28.8	1.3	2.49	4.6	3
9	3.9	16.9	28.0	1.2	2.25	3.9	3
11	3.2	16.7	28.4	1.1	2.26	3.8	3
14	4.2	16.5	28.7	1.5	2.46	3.8	3
16	3.7	16.1	29.0	1.6	2.83	7.3	3
17	3.8	16.1	29.6	1.6	2.75	9.6	3
18	3.8	16.2	33.9	1.8	2.84	8.4	3

[‡]Relative flow (1 = high water flow in at least portions of the habitat, 2 = some water flow, 3 = no water flow; Collyer 2003).
[*]Experimental pond data were summarized from a total of 1921 field measurements made between May 2001 and February 2006.
[†]Wild habitat data were pooled and summarized from Stockwell and Mulvey (1998) and Rogowski (2004). Salt Creek data represent samples collected at the middle section only. Mound Spring data were pooled from both upper and lower ponds.

collected from 26 May to 08 June in 2001 and from 06 June to 03 July 2006. Fish were live-captured with the combined efforts of beach seines and unbaited minnow traps (3 mm mesh, placed at depths less than one meter). Fish were subsequently euthanized using a lethal dose of tricaine methanesulfonate (approximately 500 mg/L Finquel® MS-222; Argent Chemical Laboratories, Inc., Redmond, WA; Summerfelt and Smith 1990) and sexed in the laboratory (Collyer et al. 2005). Digital images of each individual were used for the collection of body shape in this study. Specimens were positioned so that the left lateral side of each body was captured (Kelsch and Shields 1996; Hubbs and Lagler 2007).

One-hundred and ninety-five (out of 1120) White Sands pupfish were obtained from archived images of wild specimens collected in 2001 (Table 2.2; Collyer et al. 2005).

Table 2.2 Summary of the size and number of male and female White Sands pupfish used for analyses, including both wild populations and experimental pond populations.

	Males				Females			
	Standard Length (mm)[†]				Standard Length (mm)[†]			
Wild Habitats[*]	Min	Mean	Max.	N	Min	Mean	Max.	N
MO-2001	24.49	31.39	43.88	45	25.04	29.38	38.84	50
MO-2006	29.07	33.94	38.39	44	27.85	33.92	40.95	48
SC-2001	31.44	37.97	45.89	50	31.81	37.15	44.41	50
SC-2006	27.26	31.9	37.51	62	27.72	32.34	38.35	61
	Standard Length (mm)				Standard Length (mm)			
Experimental Ponds[‡]	Min	Mean	Max.	N	Min	Mean	Max.	N
8	27.77	34.62	44.86	53	27.19	37.05	46.06	45
9	27.76	34.72	45.27	53	27.68	34.76	42.35	48
11	28.14	34.91	43.01	50	29.56	36.63	50.53	52
14	25.65	34.39	44.62	55	26.49	32.95	44.99	53
16	27.24	36.13	48.31	50	25.54	34.19	47.82	50
17	28.54	34.49	49.74	46	26.77	33.23	46.69	53
18	28.71	36.55	47.85	55	29.21	38.28	46.66	47

[*]Mound Spring (MO) and Salt Creek (SC) populations were collected in 2001 and 2006 (Habitat and year are hyphenated for differentiating groups).
[‡]Experimental pond populations were collected in 2006.
[†]Standard length was collected digitally using geometric morphometric techniques in tpsDig v. 2.14 (available at: http://life.bio.sunysb.edu/morph/).

These fish were originally photographed in 2001 with a 1.75 megapixel Minolta RD-175® digital camera mounted on a photography stand approximately 25 cm directly above the table. Images were captured using flash lighting. All specimens were patted dry and placed on pre-scaled dull construction paper for contrast and scale (Collyer et al. 2005).

In 2006, the experiment was terminated and 925 (out of 1120) voucher specimens were preserved in a 10% formalin solution (Table 2.2). Fish were photographed in 2010; nearly 4 years later. Images of preserved specimens were collected using an 8.0 megapixel Canon PowerShot S51S® digital camera mounted on a photography table approximately

13 cm directly above the table. Images were captured using super-macro and 2 second delay settings with a combination of both flash and flood (two 120 volt, 65 watt rear oriented bulbs) lighting. Specimens were patted dry and placed on standard 3 x 5 inch ruled note cards to provide both contrast and scale. Because year 2006 fish were preserved, we were careful not to unnaturally position fish against the table surface. If necessary, probes were used to flatten the left lateral surface if curling occurred. A total of seven specimens (0.62%) were removed from the combined pupfish collections due to problems with positioning.

A set of thirteen anatomically homologous landmarks were chosen to estimate specimen size and describe overall body shape. Landmarks were collected from the left lateral body of each specimen according to Collyer et al. (2005, 2007) (Figure 2.2A). This set of landmarks was chosen because previous studies with *C. tularosa* have demonstrated that patterns of body shape were related to environmental factors (Collyer et al. 2005, 2007). All specimens were digitized from photographs of specimens using tpsDig v. 2.14 (Rohlf 2009). This program digitally stores *x*-, *y*- Cartesian coordinates, which are scaled and later transformed into shape variables.

All 1120 images were digitized by BMK to reduce potential measurement error (Arnqvist and Mårtensson 1998; Claude 2008). Measurement error was found to be small (0.075% ME) among 608 subsampled individuals (measurement error was estimated by dividing the within-subject variation by the variation found between the two groups, multiplied by 100; Appendix E- see Figure E.2 and Figure E.1).

Overall body shape was quantified for the full set of landmarks using landmark-based geometric morphometric techniques that generate shape variables free of all

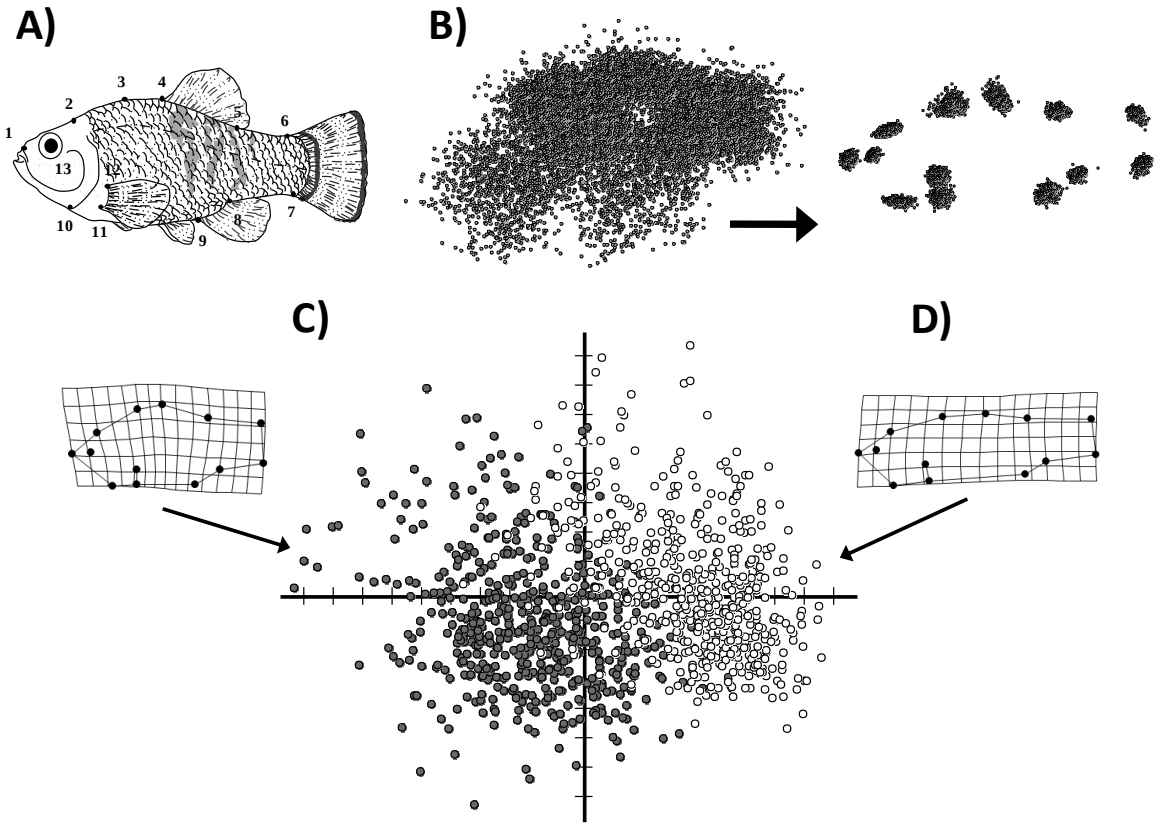

Figure 2.2 Graphical representation for quantifying shape variables from White Sands pupfish using landmark-based geometric morphometric techniques. A) quantifying raw data from 13 landmarks recorded on left lateral body of White Sands pupfish, B) before and after GPA: removing non-shape variation from landmarks of 1120 specimens, C) graphical representation of relative warp scores on the first two principal component axes: males (gray) and females (white), and D) thin-plate spline deformation grids depicting observed extremes among male specimens (left) and female specimens (right). Illustration by Brandon M. Kowalski.

nonshape variation in the absence of allometry (i.e., changes in shape associated with body size; see Appendix F) (Bookstein 1986; Rohlf and Marcus 1993; Rohlf 2000a; Adams et al. 2004). Nonshape variation was removed using least-squares generalized Procrustes superimposition methods (Rohlf and Slice 1990; Zelditch et al. 2004), which 1) centers each specimen onto a common coordinate system, 2) scales each specimen to unit centroid size, and 3) optimally rotates each specimen to a common orientation (Figure 2.2B; see

Appendix J). Male and female specimens were aligned together and later split for subsequent analyses because of documented sexual dimorphism in shape (see Figure 2.2C; Collyer et al. 2005).

From the aligned specimens, 22 shape variables were generated using the program tpsRelw v. 1.46 (Rohlf 2008) to develop a set of relative warp scores that summarize all aspects of shape variation in the data set (Rohlf 1993). Shape variables generated by this process allowed for hypothesis testing using statistical multivariate statistical procedures (see e.g., Adams and Rohlf 2000; Collyer et al. 2005; Collyer and Adams 2007).

Morphological patterns of divergence were visualized through a principal component analysis (PCA), where specimens were rigidly rotated and projected on PC axes (Figure 2.2C). Least-squares group means were generated using residuals after regressing shape against logCS to visualize shape difference with respect to a common shape allometry. Graphical deformation grids were then generated from a thin-plate spline continuous function (Bookstein 1989; Bookstein 1991) using the program tpsSplin v. 1.20 (Rohlf 2004), which graphically interpolates patterns of associated shape change among predicted group means with respect to the overall consensus body form (Figure 2.2D). Thin-plate spline deformations were accentuated by a factor of 3 to amplify shape change, and links between landmarks were included to facilitate a visual understanding of deformation among groups. All morphometric analyses were performed using TPS software (available at: http://life.bio.sunysb.edu/morph/).

Statistical Analyses

All analyses treated males and females separately based on documented sexual dimorphism in shape (Collyer et al. 2005). The significance of overall body shape

variation was tested among groups using multivariate analysis of covariance (MANCOVA) (Klingenberg 1996). Shape data were tested for effects attributable to specimen size (covariate), population, year, and all interactions. Specimen size was extracted as centroid size (CS, the square root of the summed squared distances of each landmark from the configuration centroid) and log transformed for normality (logCS) (Bookstein 1991). In this study, centroid size was highly correlated with standard length ($r \geq 0.996$, $P < 0.0001$). We approximated $F_{calc.}$ using Wilk's λ values for all sources of variation and their interactions. Models were considered significant at the 95% confidence level (i.e., $\alpha = 0.05$).

Shape differences among groups were quantified as the Euclidean distance ($D_E = (\Delta\bar{\mathbf{Y}}_i \Delta\bar{\mathbf{Y}}_i^t)^{1/2}$) between population least-squares means after accounting for a common size covariation. Significant differences between group means were found using randomized residual permutation procedures (RRPP; Adams and Collyer 2007; Collyer and Adams 2007; Adams and Collyer 2009). Randomized residual permutation procedures were performed using two linear models: 1) a full model that included size covariation (logCS), population, and year effects (including population by year interactions), and 2) a nested reduced model that included only size and year effects. This approach allowed for the evaluation of inter-population differences, while accounting for variation in shape due to size (logCS), and temporal effects (i.e., year). Permutation procedures were performed 10,000 times (including observed values) to generate distributions of pairwise distances (D_E) for determining significance. The proportion of random distances greater or equal to the observed values was treated as the significance of the observed value (Adams and Rohlf 2000; Collyer and Adams 2007). All statistical analyses and graphics were produced

in the statistical program R, v. 2.11.1 (available at: http:/www.r-project.org; R Development Core Team 2009).

Because our design allowed us to evaluate pond divergence from the ancestral and derived body shapes, we divided the observed inter-population differences into two fundamental formats: 1) *Allochronic*: comparing shape differences for the same population at different points in time, and 2) *Synchronic*: comparing shape differences among extant populations from a common origin (See Hendry and Kinnison 1999). Merging these two designs provided a unique approach for gathering novel inferences regarding the temporal divergence of the experimental populations. Aberrant comparisons (i.e., MO-2001 vs. SC-2006, MO-2006 vs. SC 2001, and MO-2001 vs. Ponds) that didn't clearly fit this format were removed from discussion.

Results

Habitat Relationships

NMDS on environmental data suggested that experimental ponds were more closely associated with Mound Spring sites than Salt Creek (Table 2.1; Figure 2.3A). While Salt Creek is a saline-lotic system, both Mound Spring and the experimental ponds are relatively brackish and lentic in nature. Despite concerted efforts to emulate the ecological features when constructing the experimental ponds, the ponds differed from the native Mound Spring habitat (Table 2.1). Differences in clustering among these habitats appeared to be most influenced by differences in temperature. Average temperatures for the experimental ponds were considerably higher (16.1-16.9 °C) than those observed at Mound Spring (6.1 °C) (Table 2.1). Biplots with and without temperature are shown in Figure 2.3.

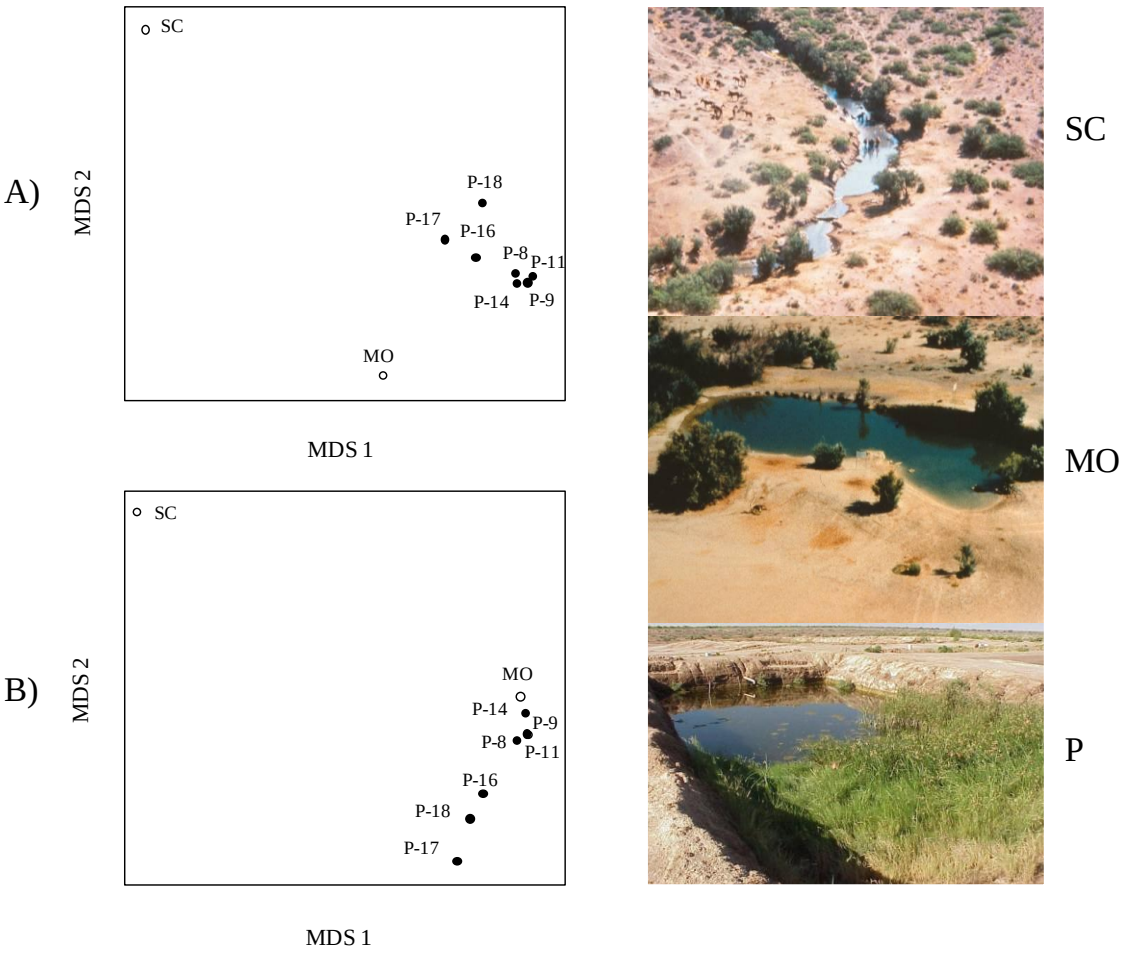

Figure 2.3 Biplots of the first two nonmetric multidimensional scales for White Sands pupfish habitats (Figure 2.1). Variables used in biplot (A) included minimum, mean, and maximum values for temperature and salinity, and ordered values of water flow (relative flow; Table 2.1). Biplot (B) included minimum, mean, and maximum values for salinity, and ordered values of water flow only. Clustering was computed using 9999 randomly started ordinations. Photographic images are indicated with the following acronyms: Salt Creek (SC), Mound Spring (MO), and experimental ponds (P). Images SC and MO were acquired from archived White Sands Missile Range photos. Image P was taken by C.A.S.

The removal of temperature effects reduced the inter-habitat distinction between Mound Spring and experimental ponds however increased the dispersion among ponds. Therefore, we concluded that the experimental ponds mimic the salinity and flow conditions of Mound Spring, but differ in temperature profiles.

Body Shape Variation

MANCOVA revealed significant body shape variation for population, year, and size effects for both males and females (Table 2.3), except for the highest interaction level term for males (Wilks' $\Lambda = 0.940$, $F_{22, 520} = 1.50$, $P = 0.0665$), indicating that all factors and most interactions are important for the morphology of White Sands pupfish.

Projections of the least-squares population means onto the first three PCs revealed divergent patterns of shape variation among populations in morphospace (Figure 2.4; Figure 2.5). We found that the first three PCs of residual variation captured 27.9%, 16.6%, and 10% of the explained variance for males; whereas, these three PCs captured 29.7%, 16.5%, and 11% of the explained variance for females, respectively. These patterns graphically confirmed the population and year differences identified in MANCOVA.

Large temporal differences were evident among wild populations. The Mound Spring population showed a substantial shift in morphospace for males (Figure 2.4) and a more subtle shift for females (Figure 2.5); whereas, the Salt Creek population showed consistently large shifts for both sexes. Furthermore, the projection of least-squares means demonstrated that experimental pond populations tended to express intermediate body shapes with respect to 2001 and 2006 Salt Creek means in morphospace.

Allochronic Comparisons

Using the randomization procedure, we found limited levels of body shape divergence for most allochronic comparisons (Table 2.4). Experimental body shape did not significantly differ for all 7 male populations and 6 of 7 female populations from their ancestral 2001 Salt Creek form. The female pond 14 was the only population with significant divergences, indicating the retention of the ancestral Salt Creek shape for almost

Table 2.3 Multivariate analysis of covariance for sources of body shape variation among wild and experimental White Sands pupfish populations, respectively for both males and females.

	Males					Females				
Source	Wilks' λ	F	Num df	Den df	P	Wilks' λ	F	Num df	Den df	P
POPULATION	0.063	9.89	176	3942	<0.0001	0.038	12.02	176	3896	<0.0001
YEAR	0.555	18.94	22	520	<0.0001	0.469	26.36	22	514	<0.0001
LOGCS	0.284	59.54	22	520	<0.0001	0.422	32.02	22	514	<0.0001
POP:YEAR	0.526	21.26	22	520	<0.0001	0.538	20.02	22	514	<0.0001
POP:LOGCS	0.553	1.83	176	3942	<0.0001	0.614	1.47	176	3897	<0.0001
YEAR:LOGCS	0.928	1.82	22	520	0.0130	0.876	3.29	22	514	<0.0001
POP:YEAR:LOGCS	0.940	1.50	22	520	0.0665	0.920	2.03	22	514	0.0039

Fcalc. was approximated using Wilk's λ values for all sources of variation and their interactions. Models were considered significant at the 95% confidence level (i.e., $\alpha = 0.05$). Significance associated with specimen size indicates that shape change is associated with fish growth, and that significant interactions among main effects associated with logCS have very different allometric patterns among groups.

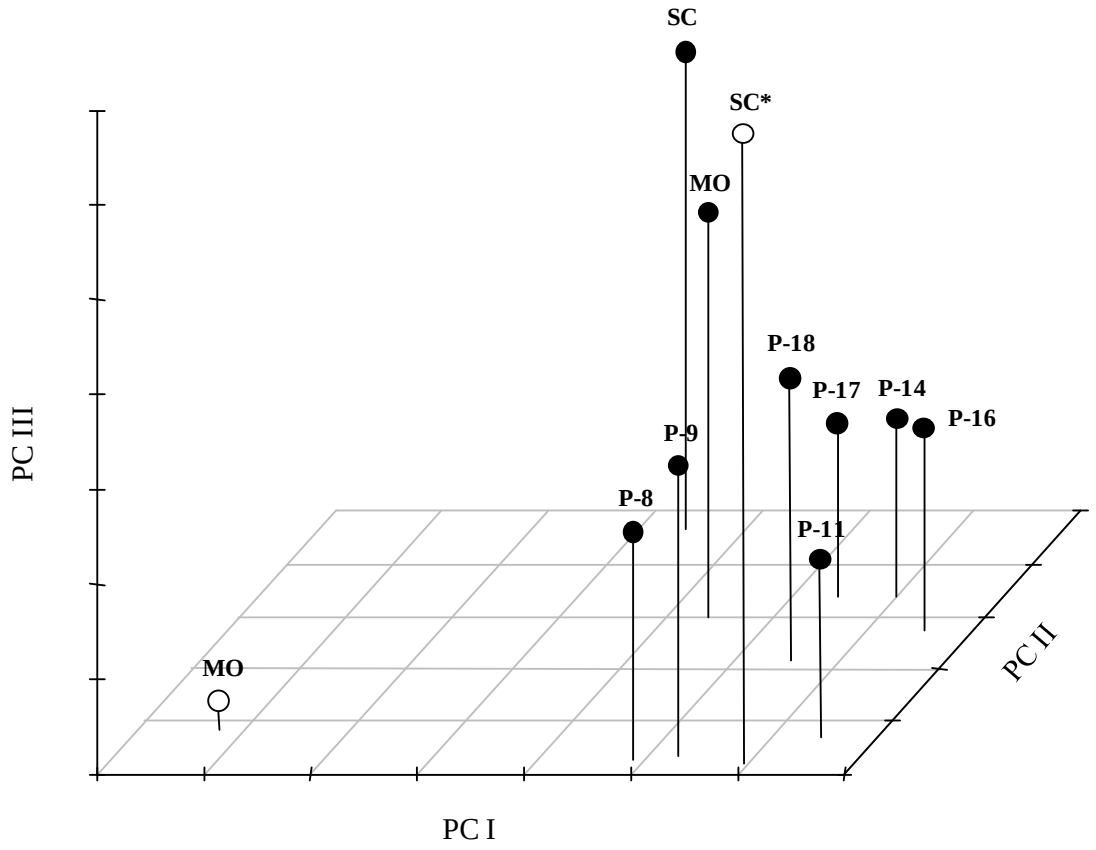

Figure 2.4 Principal component plot of shape variation for collected male White Sands pupfish. Values in plots correspond to size adjusted population means projected onto the first three PCs. Unfilled points represent year 2001, and filled points represent year 2006 White Sands pupfish collections. Asterisk indicates the founding shape mean for all experimental ponds. PC plots correspond to 54.7% of total shape variation.

all populations. Moderate levels of temporal divergence were also found for both wild populations. Significant temporal divergence was only observed for female Salt Creek (DE = 0.045) and male Mound Spring (DE = 0.023) populations, indicating substantial year-to-year variation.

Synchronic Comparisons

Euclidean distances were highly significant for almost all synchronic comparisons. Experimental populations were significantly divergent from both derived 2006 Salt Creek

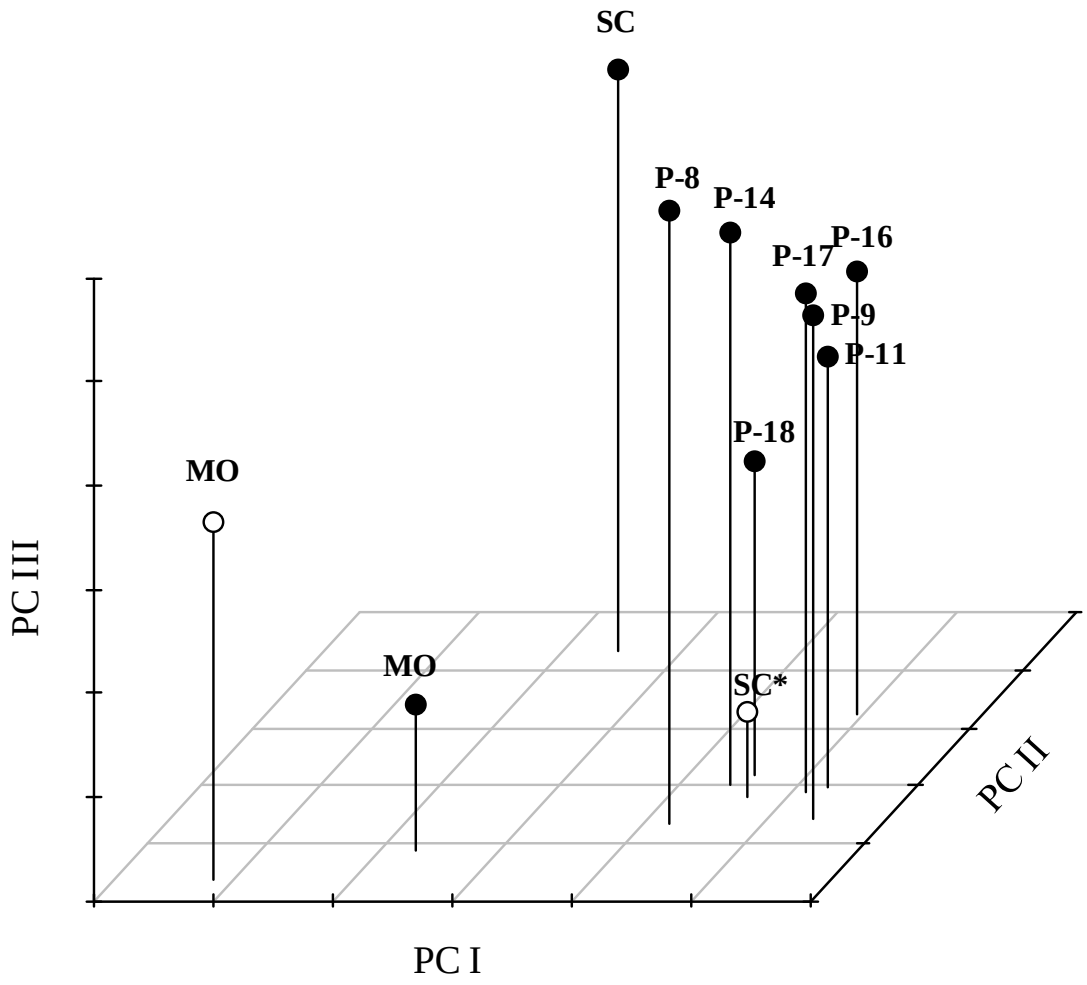

Figure 2.5 Principal component plot of shape variation for collected female White Sands pupfish. Values in plots correspond to size adjusted population means projected onto the first three PCs. Unfilled points represent year 2001, and filled points represent year 2006 White Sands pupfish collections. Asterisk indicates the founding shape mean for all experimental ponds. PC plots correspond to 57.8% of total shape variation.

and 2006 Mound Spring forms (Table 2.4). Experimental populations diverged from each other in 19 of 21 comparisons for males and 14 of 21 for females. Finally, significant inter-population differences were observed for all 4 wild habitat comparisons across corresponding years (Table 2.4), further corroborating observations by Collyer et al. (2005).

Deformations

Graphical thin-plate spline deformations revealed marked inter-population body shape differences for both sexes (Figure 2.6) ,which correspond to respective mean positions in morphospace (Figure 2.4; Figure 2.5). Overall, these shape differences appeared to be largely generated by variation in overall body depth, eye positioning, snout orientation and/or dorsal arching.

Body shapes among male wild populations collected in 2001 had prominently deeper bodies and caudal peduncles than body shapes collected in 2006. In all cases, Mound Spring pupfish exhibited marked dorsal arching and ventrally positioned snouts relative to other body forms. Respective to their 2001 and 2006 Salt Creek progenitor body shapes, male experimental populations, P-8 and P-9, exhibited considerably deeper body shapes, which did not significantly differ from each other (P_{Rand} = 0.3003; Table G.1). Similarly, P-11 developed a deeper body shape, however also exhibited terminal-dorsally positioned eyes. In contrast, experimental populations, P-14, P-16, P-17, and P-18 exhibited more dorsally-flattened body shapes.

Although less prominent, similar body deepening and caudal peduncle relationships were observed among female wild populations from 2001 to 2006. Respective to their 2001 and 2006 Salt Creek progenitor, female experimental ponds, P-8, P-9, P-11, and P-18 appeared to be much deeper bodied than ponds, P-14, P-16, and P-17. Notably, out of these populations, P-8, P-11, P-16, and P-18 also revealed terminal dorsally positioned eyes.

Table 2.4 Euclidean distances between least-squares means from the MANCOVA on shape for collected populations of White Sands pupfish.

	P-8	P-9	P-11	P-14	P-16	P-17	P-18	MO-2006	SC-2006	MO-2001	SC-2001
P-8		0.010	**0.019**	**0.023**	**0.022**	**0.020**	**0.017**	**0.023**	**0.034**		0.033
P-9	**0.017**		**0.017**	**0.021**	**0.020**	**0.020**	**0.015**	**0.026**	**0.035**		0.030
P-11	**0.019**	0.013		**0.021**	**0.014**	**0.019**	**0.020**	**0.034**	**0.042**		0.030
P-14	0.015	**0.021**	**0.019**		0.010	**0.015**	**0.019**	**0.028**	**0.035**		0.037
P-16	**0.025**	**0.025**	**0.020**	**0.018**		**0.015**	**0.019**	**0.030**	**0.039**		0.033
P-17	0.016	0.013	0.014	**0.015**	**0.017**		**0.019**	**0.026**	**0.030**		0.035
P-18	**0.021**	**0.019**	**0.014**	0.019	**0.020**	0.015		**0.022**	**0.035**		0.035
MO-2006	**0.034**	**0.040**	**0.042**	**0.036**	**0.042**	**0.037**	**0.033**		**0.029**	**0.023**	
SC-2006	**0.037**	**0.044**	**0.041**	**0.034**	**0.033**	**0.038**	**0.035**	**0.045**			0.039
MO-2001								0.033			**0.064**
SC-2001	0.033	0.030	0.026	**0.033**	0.030	0.027	0.025		**0.045**	**0.048**	

Values above diagonal are for males, values below are for females. Shading separates allochronic comparisons (gray) from synchronic comparisons *sensu* Hendry and Kinnison (1999). Aberrant comparisons were removed for clarity. Shape differences among groups were quantified as the Euclidean distance between population least-squares means after accounting for a common size covariation from the full model. Parameters for size and population effect were estimated by removing the population effect, randomizing the residuals, and re-estimating the population parameters from randomly assigned residuals. The proportion of randomly generated distances from 10,000 random pairwise distances, including the observed values, greater than or equal to the observed distance was treated as the significance level of the observed value. All significant values (experiment-wise $\alpha < 0.05$) from permutation tests are in bold font.

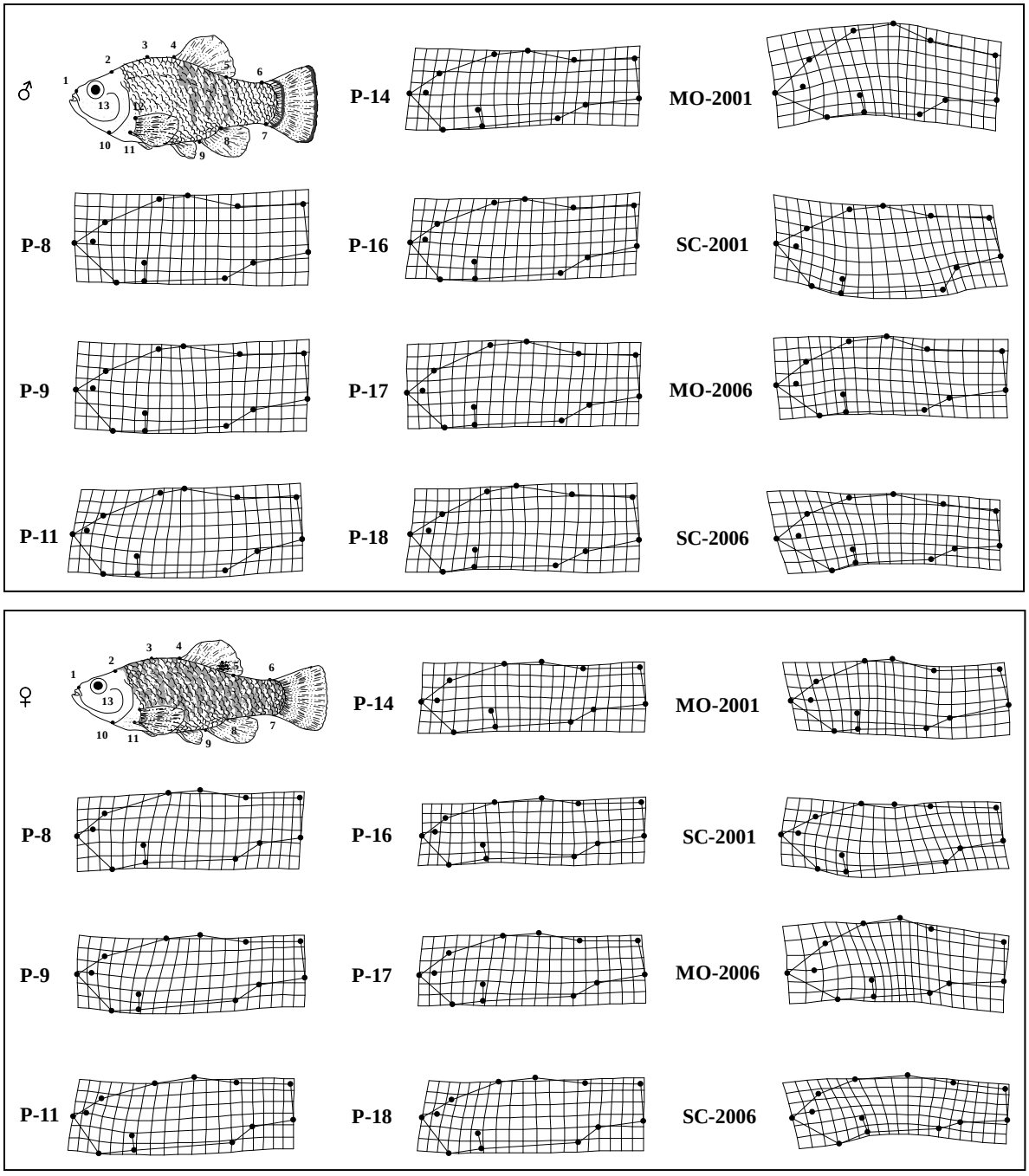

Figure 2.6 Thin-plate spline representations of group means from wild and experimental pond populations emphasizing significant shape differences for males (top panel) and females (bottom panel). Thin-plate spline deformations were accentuated by a factor of 3 to amplify shape change, and links between landmarks were included to facilitate a visual understanding of deformation among groups. Illustrations by Brandon M. Kowalski.

Discussion

The introduction of White Sands pupfish to a series of experimental ponds derived from Salt Creek led to significant body shape divergence in the wild. We predicted that morphological divergence would shift towards the Mound Spring form under a similar direction and magnitude for all experimental populations (Collyer et al. 2007). Instead, we found evidence of discordant body shapes across all principal components for both males and females, precluding any appreciable conclusion about parallel divergence. In fact, our results showed considerable body shape variation among experimental populations, indicating that divergence maybe governed by factors not measured in this study. We also observed temporal body shape differences for the wild populations at Mound Spring and Salt Creek, indicating that body shape may reflect temporal environmental variance.

The use of the allochronic/ synchronic design provided important insights into the divergence of the experimental populations. For example, had we simply examined allochronic relationships, we would have detected a non-significant outcome, leading to the conclusion that the experimental populations did not diverge. Indeed, the experimental populations appear to have retained some ancestral body shape features. This finding is in concordance to 1 year observations among the same experimental populations (Collyer et al. 2007), suggesting that these similarities may be associated with genetic constraints (Oster and Albrecht 1982; Bégin and Roff 2003). Synchronic evaluations, by contrast, indicated a marked degree of inter-population variation, and experimental populations were significantly divergent from both derived 2006 Salt Creek and 2006 Mound Spring forms following 5 years of monitoring. In part, these changes may be due to phenotypic plasticity, genetic differences, or a combination of the two (Collyer et al. 2007).

Furthermore, these discrepancies may suggest that unmeasured features or habitat intrinsic factors may have strong effects on the observed body shape variation.

Methodological constraints associated with the study design may explain the observed discrepancies between allochronic and synchronic results. For instance, the possibility exists that preservation effects may have biased estimates of allochronic divergence. That is, all 2001 samples were of photos of live fish; whereas, all 2006 samples were preserved for about 4 years prior to being photographed (*sensu* Smith and Collyer 2008).

In a separate study, we investigated the effects of formalin on body shape variation in a surrogate *Cyprinodon* species, the sheepshead minnow (*C. variegatus*; Echelle et al. 2005; see Appendix I). Body shape (RW1) was significantly affected by formalin over an 84 day study (Repeated measures ANOVA; N = 30; $F_{6,29} = 4.446$, P = 0.0003; Appendix I). Body shape deepened in response to 10% formalin (Figure 2.7; Figure I.3). Most body shape changes occurred within the first 14 days (Figure I.4), with limited changes for the subsequent 70 days.

The observed divergence in this study was larger than changes due to formalin alone, however; the global average preservation effect for the first two relative warp scores was estimated at approximately 69.55% without taking vector direction (i.e. angle differences) into account. Nevertheless, one may argue that these comparisons are limited because the study was of a surrogate species and that the experiment lasted only 84 days compared to 4 years for the current study. Various preservation effects using traditional morphometrics have been described for fish (Shetter 1936; Parker 1963; Stobo 1972; Shields and Carlson 1996; Sagnes 1997); however, to our knowledge, our study is the first

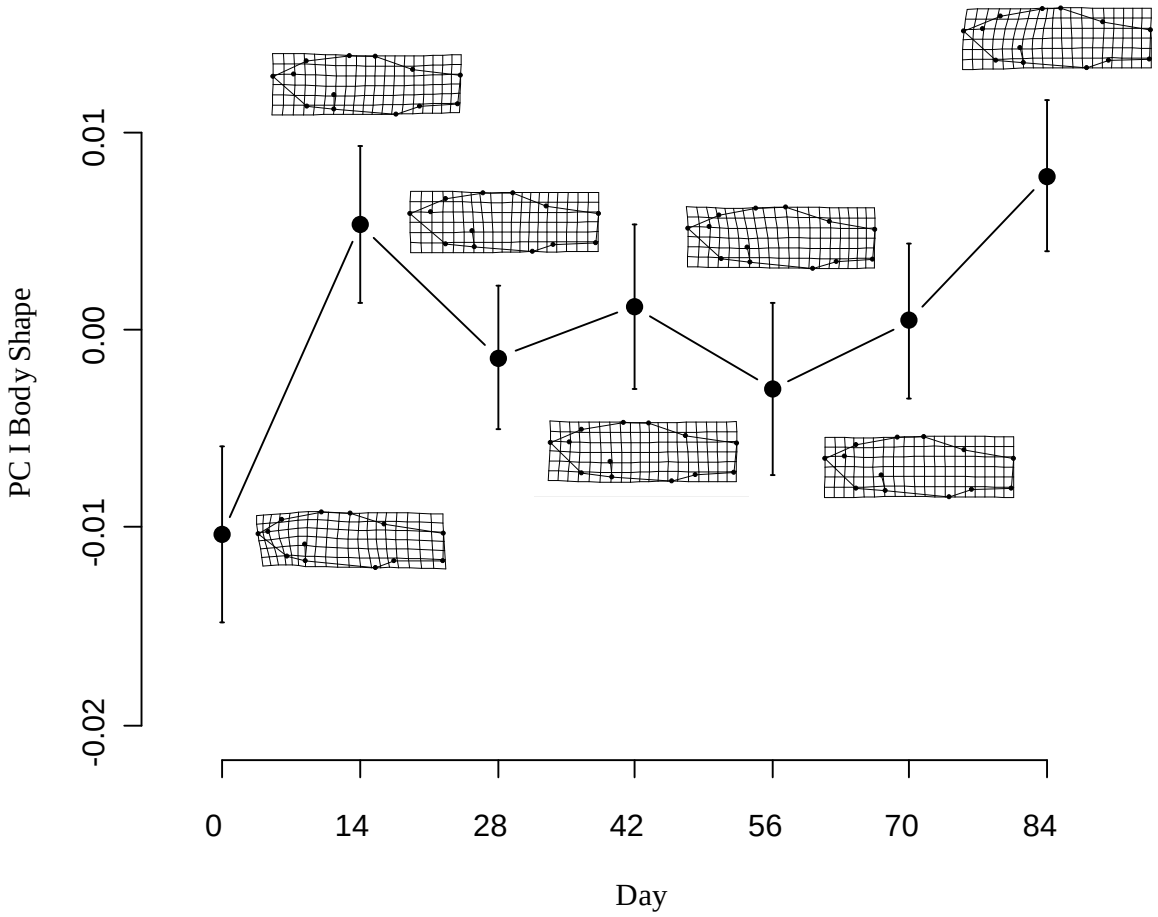

Figure 2.7 Graphical representation of shape variation among live (day 0) and preserved (days 14-84) sheepshead minnows (*C. variegatus*). Values are least-squares shape means on the first principal component (PC), representing 21.65% of the total captured variation. Bars represent the ±1 SE (N = 30). Thin-plate spline deformations were accentuated by a factor of 3 to amplify shape change, and links between landmarks were included to facilitate a visual understanding of deformation among groups.

to evaluate preservation effects on body shape. Given these observations, we have cautioned that allochronic comparisons are inherently limited due to potential temporal comparisons confounded by preservation effects. Thus for the remainder of this paper, we emphasis only synchronic comparisons, which are not confounded by preservation effects.

In the current study, population sizes varied widely among ponds, sometimes by an order of magnitude (Stockwell; Unpublished data), suggesting that the ponds were

ecologically dissimilar. We found emerging evidence of a positive relationship between fish density (derived from a mark-recapture study performed in 27 February – 03 March 2005; see Table 2.5) and all aspects of body shape (arithmetic mean) by performing a two-block partial least-squares analysis (see Rohlf and Corti 2000). Although insignificant, there was a strong positive correlation for both male ($r_{pls} = 0.721$; $P_{rand} = 0.749$) and female ($r_{pls} = 0.809$; $P_{rand} = 0.252$) populations. Emerging patterns of covariation found between data sets conveyed differences in body depth; mean body depth of fish increased in response to high population density (Figure 2.8). We argue that this observation is particularly noteworthy and suggestive of density-dependent effects on body shape.

Indirect evidence for density-dependence among White Sands pupfish was documented by Rogowski et al. (2006). Rogowski et al. (2006) reported a negative size by density associations for the Lost River population (Figure 2.1); however, few studies have directly investigated body shape differences in response to density (Svanbäck and Persson 2009). Studies with Eurasian perch (*Perca fluviatilis*) have shown that the fitness landscape of perch changes with experimental manipulation of population density (Svanbäck and Persson 2009). Deeper-bodied perch were observed under low density conditions. Although this finding is inconsistent with our results, the changes in perch were attributed to a plastic response in habitat and diet choice, depending on competitive interactions (Persson et al. 2000; Svanbäck and Persson 2004; Svanbäck et al. 2008). In our study, it is unknown whether the emergent density-dependent relationship is a result of competition for habitat (e.g., see Collyer et al. 2005), food resources, or both. It is possible that body shape may be governed by other factors (e.g., see Collyer et al. 2005, 2007).

Table 2.5 Summary of experimental population estimates from mark-recapture study performed in Winter 2005.

	n[b]		
Pond[a]	95% LCL	Mean	95% UCL
8	3380	3968	4740
9	1141	1476	2003
11	1719	2093	2620
14	2002	2117	2251
16	199	234	289
17	1166	1205	1250
18	2579	2900	3299

[a]Ponds were setup during March 2001 at the Holloman Air Force Base (HAFB), New Mexico. Two-hundred White Sands pupfish (100 male and 100 female, Young-of-the-year) were introduced from Salt Creek to each experimental pond.
[b]Noremark population estimates (n) with 95% confidence limits (CL; U, upper; L, lower) are from a mark-recapture study performed 27 February - 03 March 2005 (Stockwell, unpublished data). Data summarized from the program Mark(c) (White and Burnham 1999).

Observed changes in natural pupfish populations to environmental perturbations suggest that pupfish numbers are intrinsically linked to habitat size and available resources (Deacon and Deacon 1979). Although we did not measure resource abundance, it is reasonable to believe that our experimental ponds may have differed in resource type due to the observed differences in population size generated over time. Such a hypothesis is supported by the observed differences in eye position and body depth among experimental ponds. Similar morphological differences due to trophic associations have been documented throughout the literature (Robinson and Wilson 1995; Carpenter 1996; Caldecutt and Adams 1998; Rüber and Adams 2001; Trapani 2003; Jastrebski and Robinson 2004). In Eurasian perch, food type was found to be significantly associated with body type. Perch feeding on a larger proportion of zooplankton were more streamlined than perch feeding on larger proportions of macroinvertebrates (Hjelm et al.

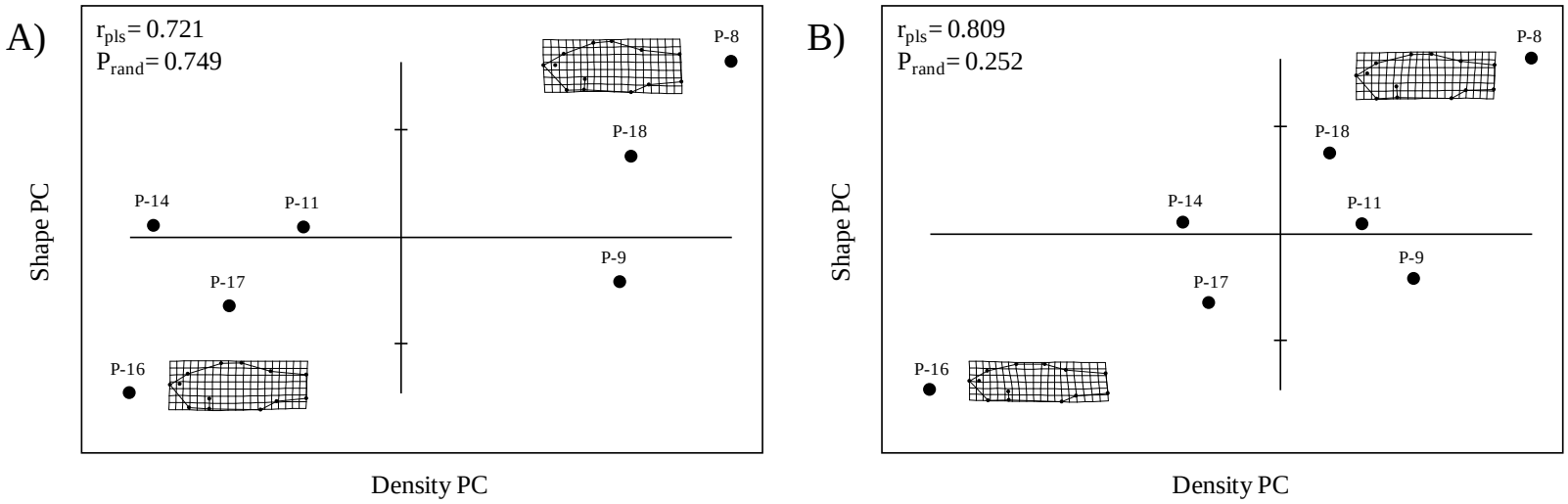

Figure 2.8 Pattern of covariation within the 2B-PLS dimension represented by density and shape variables. Plot includes population density by all aspects of body shape for experimental A) male and B) female White Sands pupfish populations. Correlation is 0.721 and 0.809 for males and females, respectively. Significance was determined by performing 9999 random permutation procedures. The proportion of observations greater or equal to the observed value was considered the significance level. Deformations of the average shape (x3) represent the range of shape change within the single 2B-PLS dimension.

2001; Svanbäck and Eklöv 2002). Furthermore, experiments conducted with populations of dace (*Phoxinus eos*) found that deeper-bodied fish were more adept at capturing evasive prey than shallow-bodied conspecifics (Toline 1993), which body shape was later found to be heritable (Toline and Baker 1997). Experiments with Atlantic cod (*Gadus morhua*) similarly found direct temperature and food supply associations. Deeper body and head shapes resulted under both higher temperature and food level rearing conditions (Marcil et al. 2006). Given that temperatures also differed slightly between our experimental populations (Table 2.1), the observed divergence may also reflect differences in temperature among ponds.

Moreover, empirical data suggest that density-linked cannibalism may influence the dynamics of a given population (Dionne 1985; Persson et al. 2000). Pupfishes lack direct parental care and are generally known to cannibalize eggs and young, especially in high density populations (Liu 1969; Soltz 1974; Loiselle 198; McMahon and Tash 1988; Deacon et al. 1995). In dense populations, intra-guild predation is bound to increase (Meffe 1984), and deeper body shapes may reduce the risk of being eaten (Brönmark et al. 1992; Eklöv and Jonsson 2007). Studies have shown that increased body depth may influence predator strikes (Webb 1986), handling time for predators (Nilsson and Brönmark 2000), and evasive performance in prey (Langerhans et al. 2004). As a result, increased body depth may provide refuge from gape-limited predators (Hambright et al. 1991; Gido et al. 1999).

Based on Collyer et al. (2005, 2007), salinity and flow appear to be important, yet the considerable temporal variation within the experimental populations would suggest that body shape responds to factors other than salinity and flow. For example, increased

numbers of White Sands pupfish from Lost River were found to be significantly correlated with more shallow-saline areas (Rogowski et al. 2006). If density covaries with salinity (a factor known to fluctuate through time), then some of the observed patterns reported by Collyer et al. (2005, 2007) may also reflect density-dependence.

The morphological shifts that occurred in our study clearly illustrate how difficult it is to construct artificial refugia that mimic natural conditions in the wild. This is of particular concern because contrasting habitats may influence the intrinsic integrity of the refuge populations, and differences in habitat characteristics imposing novel selective pressures on refuge populations may limit their usefulness as refugia (e.g., see Sharpe et al. 1973; Williams 1977; Deacon et al. 1995; Wilcox and Martin 2006). It seems reasonable to conclude that the observed divergence, although non-repeatable in this study, may have implications for the conservation of White Sands pupfish and other imperiled fishes.

The results of this study warrant the careful investigation of additional intrinsic environmental factors that may influence the expression of phenotypes. The species recovery plan for *C. tularosa* calls for the establishment of refuge populations for the Salt Creek and Malpais Spring ESUs (Stockwell et al. 1998); however, to this date only the Salt Creek population has been "successfully" replicated among refugia within the Tularosa Basin (Pittenger and Springer 1999). Conservation efforts have only recently shifted the focus toward replicating the single Malpais Spring population (Pittenger 2010). These efforts include plans to translocate Malpais Spring fish to suitable habitats in hopes to render the White Sands pupfish less vulnerable.

Our work with White Sands pupfish shows that much can be learned from the introduction of population to novel environments. We have demonstrated that refuge

populations present ideal experimental conditions, and that significant phenotypic divergence can occur from novel translocations within a short period of time. Thus, ideal conservation practices could be achieved by merging recovery efforts with experimental interest (Stockwell and Leberg 2002). However, we were unable to identify which environmental factors strongly influenced the phenotypic response among our experimental populations. Further investigations will be needed to understand which factors significantly affect body shape. Identifying the important resources or factors that affect phenotypic expression (Watters et al. 2003) may help reduce the observed differential selection that could conspire against well intended efforts (e.g., see Wilcox and Martin 2006). In the mean time, continued monitoring may be a required approach for managing rare species as the combined knowledge obtained from current translocation events in the wild will prove valuable for future planning.

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APPENDIX A. THESIS METHODOLOGY: DATA COLLECTION AND THE QUANTIFICATION OF BODY SHAPE

The left lateral body surface among White Sands pupfish was quantified using geometric morphometric techniques (Rohlf and Marcus 1993, Richtsmeier et al. 2002, Adams et al. 2004, Zelditch et al. 2004). A total of 2160 (1076 ♂ and 1084 ♀) White Sands pupfish from collection years 2001, 2002, and 2006 were sampled for the preparation of this disquisition (Table B.1). Consequently, only one-hundred-and-ninety-five (95 ♂ and 100 ♀) White Sands pupfish from year 2001 and nine-hundred-and-twenty-five (468 ♂ and 457 ♀) from year 2006 were utilized for the description of body shape divergence in Chapter 2 (Table 2.2). Nonetheless, because ancillary materials utilizing all specimens are provided in the appendices, a full description of the methods used is provided below.

Specimens collected in year 2001were sacrificed in approximately 500 mg/L tricaine methanesulfonate (Finquel® MS-222; Argent Chemical Laboratories, Inc., Redmond, WA; Summerfelt and Smith 1990), placed in ice baths and immediately photographed within 2 hours. Photographs were captured with a 1.75 megapixel Minolta RD-175® digital camera mounted on a photography stand approximately 0.25 m directly above the table using flash lighting (Collyer et al. 2005). Specimens collected in year 2002 were "euthanized" as described above, patted dry, and immediately photographed using a 3.3 megapixel "Canon" Powershot G1® digital camera (Collyer 2003; Collyer et al. 2007;

Errata: “Olympus” Powershot G1, “Anesthetized”). Year 2001 and 2002 specimens were placed on pre-scaled dull construction paper for contrast and scale.

Museum specimens from year 2006 collections were sacrificed as described above and preserved in a 10% formalin solution until photographed December 2010. Images of specimens were captured using an 8.0 megapixel Canon PowerShot S51S® digital camera mounted 13 cm directly above a photography table using super-macro and 2 second delay settings, with a combination of a flash and flood (2 x 120 volt, 65 watt rear oriented bulbs) lighting. Year 2006 Specimens were placed on standard 3 x 5 inch ruled note cards to provide both contrast and scale. Probes were used to flatten the left lateral surface if curling occurred.

For all collections, sex was recorded. Males were identified by the presence of a prominent dorsal/nuchal crest, metallic blue coloration, yellow-orange coloration along paired fins, and a black terminal band on the caudal fin (Miller and Echelle 1975). Females were identified by the presence of striated bars along their flanks and pigmentation (i.e., ocellus) among posterior dorsal membranes. Unidentifiable fish were removed from all analyses (representing 0.002 – 0.004% of the total collection).

Thirteen anatomical landmarks were selected to quantify body shape (Figure A.1 and Table A.1). These landmarks were chosen because previous studies in *Cyprinodon tularosa* have demonstrated that patterns of variation in body depth were related to ecological differences between introduced and natural habitats (Collyer et al. 2005; see Figure E.4and Figure E.3 for differences between measurers). Specimens were digitized using tpsDig v. 2.14 (Rohlf 2009a) and further manipulated for subsequent analyses using tpsUtil v. 1.44 (Rohlf 2009b). Measurement error was estimated and found to be small

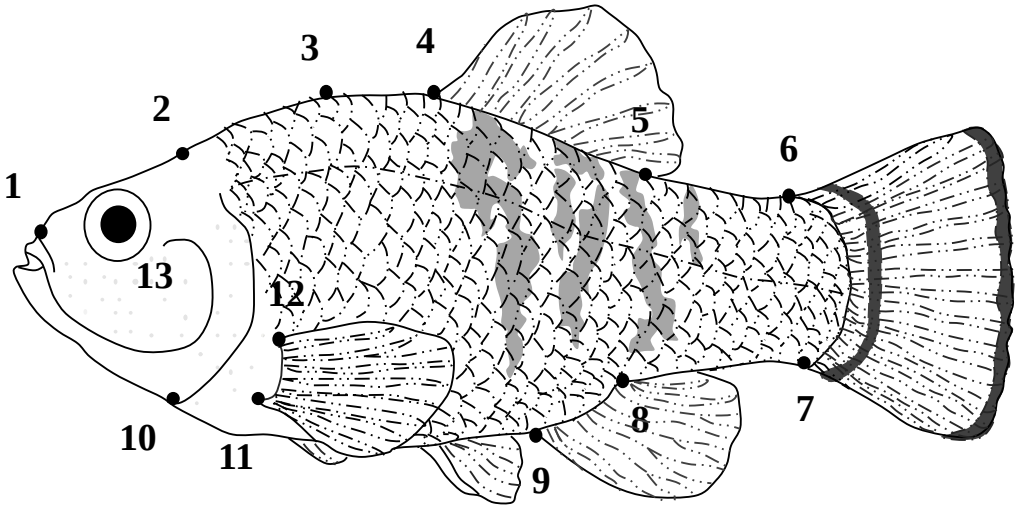

Figure A.1 Illustration of an adult male White Sands pupfish and the placement of the 13 landmarks used to capture body shape. All landmarks were placed on the left lateral body surface: 1) maxilla/neurocranium junction, 2) occiput (posterior edge of neurocranium), 3) dorsal/ nuchal crest, 4) anterior dorsal fin insertion, 5) posterior dorsal fin insertion (dorsal body/caudal junction), 6) dorsal caudal fin insertion, 7) ventral caudal fin insertion, 8) posterior anal fin insertion, 9) anterior anal fin insertion, 10) ventral subopercular/ interopercular junction, 11) ventral pectoral fin insertion, 12) dorsal pectoral fin insertion, and 13) center of eye. Illustration by Brandon M. Kowalski.

Table A.1 Typology of landmarks used for geometric morphometric analyses of White Sands pupfish.

Landmark	Type[†]	Description
1	I	Maxilla/neurocranium junction
2	II	Occiput (posterior edge of neurocranium)
3	II	Dorsal/ nuchal crest
4	I	Anterior dorsal fin insertion
5	I	Posterior dorsal fin insertion (dorsal body/caudal junction)
6	I	Dorsal caudal fin insertion
7	I	Ventral caudal fin insertion
8	I	Posterior anal fin insertion
9	I	Anterior anal fin insertion
10	I	Ventral subopercle/interopercle junction
11	I	Ventral pectoral fin insertion
12	I	Dorsal pectoral fin insertion
13	II	Center of eye

[†]Type represents Fred Bookstein's (1991) landmark classification (see Appendix J). Table modified from Collyer (2003) with permission.

(%ME= 0.075) among 608 subsampled individuals (Appendix E- see Figure E.2, Figure E.1).

All shape data were transformed by removal of size variation using least-squares generalized Procrustes analysis and statistically analyzed following the descriptions in Appendix J (Rohlf and Slice 1990). Relative warps scores were generated from the partial warps of aligned data (i.e., superimposed objects) from relative warp score analyses to develop a set of variables that summarized all aspects of shape variation in the dataset (refer to Figure 2.2). All geometric morphometric procedures were performed using tpsRelw v. 1.46 (Rohlf 2008). All TPS software is available at the SUNY at Stony Brook morphometrics website (http://life.bio.sunysb.edu/morph/), written and maintained by F. J. Rohlf.

Morphological patterns of divergence were visualized through a principal component analysis (PCA), where least-square group means, split by sex (Collyer et al. 2005), were rigidly rotated and projected on PC axes (*sensu* Figure 2.2C; See Appendix J). Thin-plate spline deformation grids for representative group means were then generated from a thin-plate spline procedure (Bookstein 1991, Bookstein 1989) to visualize patterns of associated shape change (refer to Figure 2.2; See Appendix J). Shape deformations were produced from means of aligned coordinates using tpsSplin v. 1.20 (Rohlf 2004). Thin-plate spline deformations depict shape differences from one group mean relative to another using bent grid lines similar to D'arcy Thompson's proposed transformation grids (Thompson 1917). Thin-plate spline deformations were accentuated by a factor of 3 to amplify shape change, and links between landmarks were included to facilitate a visual understanding of deformation among groups.

Phenotypic variation was examined using generalized linear models such that:

$$\boldsymbol{Y} = \mathbf{X}\boldsymbol{\beta} + \boldsymbol{\varepsilon}, \qquad \text{Eq. A.1}$$

where, **Y** is the full set of 2K-4 (K being the number of landmarks) shape data, **X** is the design matrix containing logarithmic centroid size (covariate), population, year, and all interactions, **β** is the coefficient matrix, and **ε** is the matrix of residuals. Third order polynomial models were briefly explored; however, models did not appear to improve predictions (M L Collyer, Pers. Comm.).

The significance of overall body shape variation was tested among groups using multivariate analysis of covariance (MANCOVA). Shape data were tested for effects attributable to logarithmic centroid size (covariate), population, year, and all interactions. All tests of significance were based on type I (sequential) sum-of-squares. $F_{calc.}$ was approximated using Wilk's λ values for all sources of variation and their interactions. Models were considered significant at the 95% confidence level (i.e., $\alpha = 0.05$).

The significance of the differences between group means was found using randomized residual permutation procedures (RRPP; Adams and Collyer 2007, Collyer and Adams 2007; Adams and Collyer 2009). This approach allowed for the evaluation of inter-population differences, while accounting for differences in both allometries (using logarithmic centroid size), and temporal effects (i.e., a year effect).

The randomized residual permutation procedure was performed using two linear models: 1) a full model that included size covariation (geometric size), population, and year effects (including all interactions), and 2) a reduced model included only size and year effects (and their integrations). These models allowed for the evaluation of population effects while holding both size covariation and year effects constant.

Least-squares means were calculated from the full model above and used for visualizing patterns of morphological patterns were graphically visualized along PC axes. Group mean differences were quantified by calculating the Euclidean distances between groups; where, the Euclidean distance between means was calculated as:

$$D_i = (\Delta\overline{\boldsymbol{Y}}_i \Delta\overline{\boldsymbol{Y}}_i^t)^{1/2}, \quad \text{Eq. A.2}$$

where,

$$\Delta\overline{\boldsymbol{Y}}_i = \overline{\boldsymbol{Y}}_1 - \overline{\boldsymbol{Y}}_2, \quad \text{Eq. A.3}$$

where *i* is the *i*th Euclidean distance between group means 1 and 2, and t corresponds to the transpose of the vector of 2K -4 means (K being the number of landmarks).

Residual phenotypic values from the reduced model were shuffled to create a situation where the null hypothesis is true (i.e., no difference among groups), while holding size and year effects constant. Randomized residuals ($^*\boldsymbol{\varepsilon}_r$) of reduced model were added to the predicted values $\left(\widehat{\mathbf{Y}}_r\right)$ of the reduced model to produce random values ($^*\mathbf{Y}$) such that,

$$\widehat{\boldsymbol{\beta}}_r = (\mathbf{X}^t\mathbf{X})^{-1}\mathbf{X}^t\mathbf{Y}, \quad \text{Eq. A.4}$$

$$\widehat{\mathbf{Y}}_r = \mathbf{X}\widehat{\boldsymbol{\beta}}_r, \quad \text{Eq. A.5}$$

$$\boldsymbol{\varepsilon}_r = \mathbf{Y}_r - \widehat{\mathbf{Y}}_r, \quad \text{Eq. A.6}$$

and

$$\mathbf{Y}^* = \mathbf{Y}_r + \boldsymbol{\varepsilon}_r^*, \quad \text{Eq. A.7}$$

where r designates values calculated from the reduced model, $\boldsymbol{\beta}$ is a kxp and the symbols ^, t, -1, and * correspond to the predicted values, matrix transpose, inverse, and new values respectively. The full model was then used to calculate predicted values from the randomized data (**Y***). From these values, randomized least-squares means were calculated and new Euclidean distances estimated. Repeating this procedure many times produced an

empirical null distribution of Wilk's Λ for α (Collyer and Adams 2007), which was used for testing significance among populations, without neglecting size (i.e., centroid size covariation) and year effects (i.e., the reduced model nested within the full model creates a situation where the added randomized residuals to the predicted effects, preserves size, year, and their interactions). The proportion of random distances greater or equal to the observed values was treated as the significance level. All statistical analyses and graphics were performed using the statistical program R, v. 2.11.1 (available at: http:/www.r-project.org; R Development Core Team 2009). R code for randomized residual permutation procedures can be obtained from Dr. Dean Adam's web site (available at: http://www.public.iastate.edu/~dcadams/).

APPENDIX B. THE COMPLETE WHITE SANDS PUPFISH COLLECTION

This section summarizes the total number of individual fish sampled from each population over 3 collection years. A grand total of 2160 White Sands pupfish were digitized by BMK using landmark-based geometric morphometric techniques (Table B.1). Only a subset of these samples was used for shape analyses in Chapter 2; however, because many ancillary materials and analyses in the following appendices incorporate the additional samples, a summary of all digitized samples is provided below.

Six experimental ponds out of 15 created were founded with fish from Lost River (Table D.1). Among these populations, P-13 was the only sampled experimental pond founded by the Lost River population. Because there was insufficient replication of Lost River derived populations, pond 13 was also removed from all analyses.

Erosive problems occurred between ponds 11 and 12 early into the experiment; where, subterranean erosion connected ponds 11 and 12. Despite these complications, pond 12 was never originally stocked with fish. As a result, ponds 11 and 12 were treated as one pond. Mark-recapture work show that fish moved freely between ponds 11 and 12 (C A Stockwell, Pers. Comm.). Collections made in 2002 were sampled from the original pond 11; whereas, fish were sampled from pond 12 in 2006.

Table B.1 Number of female and male White Sands pupfish digitized by BMK from wild and experimental populations collected in 2001, 2002, and 2006.

	2001		2002		2006		
Wild Populations	Females	Males	Females	Males	Females	Males	Total
Lost River	50	46	50	50	-	-	*196*
Malpais Spring	50	49	50	50	-	-	*199*
Mound Spring	50	45	49	49	48	44	*285*
Salt Creek	50	50	50	50	61	62	*323*
Total	*200*	*190*	*199*	*199*	*109*	*106*	*1003*

		2002		2006		
Experimental Populations	Source[‡]	Females	Males	Females	Males	Total
P-8	SC	24	26	45	53	*148*
P-9	SC	25	25	48	53	*151*
P-11[†]	SC	24	25	52	50	*151*
P-13*	LR	25	25	47	51	*148*
P-14	SC	25	25	53	55	*158*
P-16	SC	-	-	50	50	*100*
P-17	SC	25	25	53	46	*149*
P-18	SC	25	25	47	55	*152*
Total		*173*	*176*	*395*	*413*	*1157*

[‡]Source indicates translocation history for experimental ponds, Salt Creek (SC) or Lost River (LR).
*Samples that were digitized, but excluded from supplementary shape analyses.
[†]Pond 11 eroded into pond 12. Pond 12 was never stocked with Fish, thus both ponds 11 and 12 were treated equally as Pond 11. Fish sampled in 2002 were collected from the original pond 11. Fish collected from 2006 were collected from pond 12.

APPENDIX C. FOUNDING EXPERIMENTAL PUPFISH DATA

Table C.1 Summary of data for founding Lost River and Salt Creek White Sands pupfish among experimental ponds.

Female White Sands pupfish						
			Mass (g)		Standard Length (mm)	
Source	Pond	N	Mean	± 95% CL	Mean	± 95% CL
SC	8	100	0.650	0.048	26.765	0.749
SC	9	100	0.489	0.046	24.871	0.813
LR	10	100	0.170	0.012	18.447	0.401
SC	11	100	0.548	0.043	26.097	0.730
LR	13	99	0.225	0.015	19.615	0.459
SC	14	100	0.589	0.043	26.039	0.674
LR	15	100	0.218	0.013	20.615	0.387
SC	16	100	0.536	0.046	25.471	0.835
SC	17	100	0.477	0.043	25.337	0.794
SC	18	100	0.476	0.046	25.358	0.799

Male White Sands pupfish						
			Mass (g)		Standard Length (mm)	
Source	Pond	N	Mean	± 95% CL	Mean	± 95% CL
SC	8	100	0.754	0.034	28.172	0.381
SC	9	100	0.721	0.029	27.909	0.348
LR	10	100	0.320	0.018	22.210	0.345
SC	11	100	0.723	0.033	27.974	0.394
LR	13	100	0.344	0.019	22.662	0.411
SC	14	100	0.708	0.041	27.361	0.546
LR	15	100	0.372	0.018	23.484	0.421
SC	16	100	0.750	0.029	27.961	0.337
SC	17	100	0.730	0.034	27.472	0.370
SC	18	100	0.757	0.030	27.951	0.321

Source indicates translocation history for experimental ponds, Salt Creek (SC) or Lost River (LR). Ponds were setup during the March 2001 at the Holloman Air Force Base (HAFB), New Mexico and founding fish were established in late May and early June 2001.

APPENDIX D. THE FATE OF EXPERIMENTAL PONDS

Table D.1 Fate of White Sands pupfish experimental ponds.

Salt Creek Derived		Lost River Derived		Student Ponds*	
Pond	Fate	Pond	Fate	Pond	Fate
P-3	Extinct	P-5	Extinct	P-1*	Empty
P-4	Extinct	P-6	Extinct	P-2*	Empty
P-8	Persisted	P-7	Extinct	P-12*	Empty
P-9	Persisted	P-10	Persisted		
P-11	Leaked into P-12	P-13	Persisted		
P-14	Persisted	P-15	Extinct		
P-16	Persisted				
P-17	Persisted				
P-18	Persisted				

*Experimental ponds P-1, P-2 and P-12 were never stocked with fish and left vacant for additional student experiments. In September 2001 experimental pond P-11 connected to the empty pond P-12 due to subterranean erosion during a flood event. In 2006, all experimental ponds were terminated and voucher samples from each pond were preserved to evaluate body shape divergence.

APPENDIX E. THE REPEATABILITY AND PLACEMENT OF LANDMARKS

In studies were subtle morphological variation is being tested (e.g., inter-population differences within the same species), measurement error can be a serious problem (Arnqvist and Mårtensson 1998). Measurement error is defined as "the variability of repeated measurement of a particular character taken on the same individual, relative to its variability among individuals (Bailey and Byrnes 1990)" and may come from many sources. This includes error due to: 1) the measurement device, 2) definition of the measure, 3) quality of the measured material, 4) the measurer, 5) the environment of the measurer, and 6) the measurement protocol (Lee 1990; Yezerinac et al. 1992; Claude 2008). Because statistical models typically partition explainable and residual variation, high levels of measurement error may reduce statistical power by increasing type II error (i.e., failure to reject the null when it is false); diluting predicted trends and patterns by skewing residual variances (Arnqvist and Mårtensson 1998).

Percent measurement error was estimated by dividing the squared within-subject variation by the squared variation found between replicates, multiplied by 100 (M L Collyer, Pers. Comm.). Due to time constraints, only a subsample of individuals was selected. Estimates of measurement error were made on 608 White Sands pupfish sampled from Salt Creek and Mound Spring populations collected in years 2001, 2002, and 2006 (See Table B.1). Both Salt Creek and Mound Spring populations were selected *a priori* to maximize the observed variation in the sampled data set, because measurement error is largely influenced by the measure of inter-specimen dispersion (i.e., within S^2/ between S^2). Repeated measures of each individual were digitized by the same measurer (BMK).

This whole procedure was repeated twice, where each individual was represented by one photographic image. This process removed measurement error due to methodological and instrument error.

Data collected by BMK was also compared to former data published by Collyer et al. (2005, 2007; herein referred to as MLC) to evaluate differences between studies. A total of 935 White Sands pupfish subsampled from years 2001 and 2002 were compared. Each individual was represented by the same photographic image. Data were measured once by each measurer.

A two-block partial least squares analysis (2B-PLS) was performed to explore patterns of covariation between the two sets of shape variables. This method constructs new variables (via SVD; see Appendix J) by maximizing the covariation between the two original sets of variables. From these new variables, a partial least-squares correlation (r_{pls}) that incorporates all aspects of shape variation of both data sets was determined (Rohlf and Corti 2000; Bastir and Rosas 2005). Significance was determined by performing 9999 random permutation procedures. The proportion of observations greater or equal to the observed value was considered the significance level. Two-block partial least square results were generated using tpsPLS v. 1.18 (Rohlf 2006). Mean landmark configurations were plotted to visualize differences between replicates. Means were plotted using tpsRelw v. 1.46 (Rohlf 2008). All TPS software is available at: http://life.bio.sunysb.edu/morph/.

Measurement error was found to be small (0.075% ME) between the two sets of shape variables measured by BMK. A 2B-PLS analysis revealed a significant positive relationship between the two data sets for all 22 shape dimensions ($P_{rand} \leq 0.0001$). The correlation within the first 2B-PLS dimension is 0.985 (Figure E.1). Small differences

between the two sets of shape variables were indicated (Figure E.2). The largest deviations occurred at landmarks 3 and 5.

In addition, 2B-PLS analysis revealed a strong positive relationship between the two data sets measured by BMK and MLC for all dimensions ($P_{rand} \leq 0.0001$). The correlation within the first 2B-PLS dimension is 0.9787 (Figure E.3). Apparent deviations occurred at landmarks 2, 3, 5, and 10 (Figure E.4).

These observed discrepancies were likely due to the variability in locating discrete anatomical juxtapositions within some specimens and/or differences between respective measurers. For instance, landmark 3 (Dorsal/ nuchal crest) is classified as a type II landmark (Bookstein 1991; see Appendix J), which is found by locating the maxima of a curvature within an object; whereas, landmark 5 (Type I landmark; Posterior dorsal fin insertion) most likely accrued differences due to folding in the dorsal fin. Comparing data sets from BMK to MLC, larger discrepancies accrued. Indeed, the measurement protocols and environments between measurers likely differed, which predictably introduced a potential source of error (Claude 2008). These data suggest that despite the observed discrepancies, shape data were found to be repeatable.

Repeatability of Shape Measurements

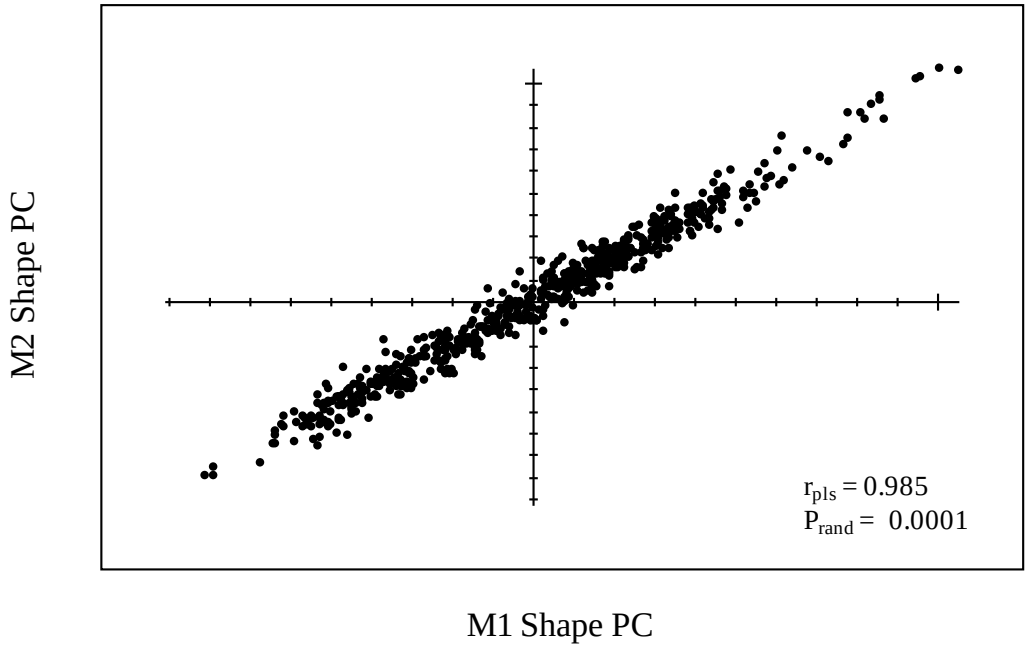

Figure E.1 Plot of first 2B-PLS dimension illustrating a strong relationship between 608 White Sands pupfish from wild habitats Salt Creek and Mound Spring sampled across all years (2001, 2002, and 2006). Measurement error was quantified from samples of the same image that were replicated twice (M1 & M2). All samples were digitized by BMK. Significance was determined by performing 9999 random permutation procedures. The proportion of observations greater or equal to the observed value was considered the significance level.

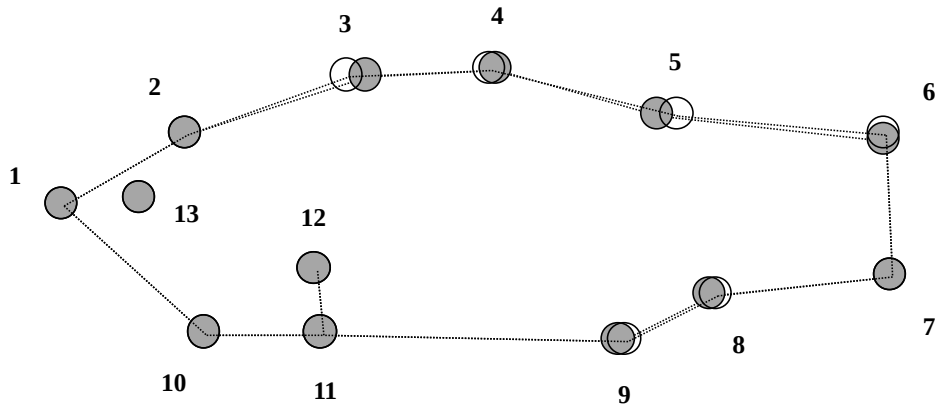

Figure E.2 Consensus shapes depicting the overall measurement error of 13 anatomical landmarks placed on 608 White Sands pupfish by BMK from Salt Creek and Mound Spring sampled across all years (2001, 2002, and 2006). Measurement error was quantified from samples of the same image that were replicated twice. Percent measurement error is 0.075. The largest observed deviations occurred at landmarks 3 and 5.

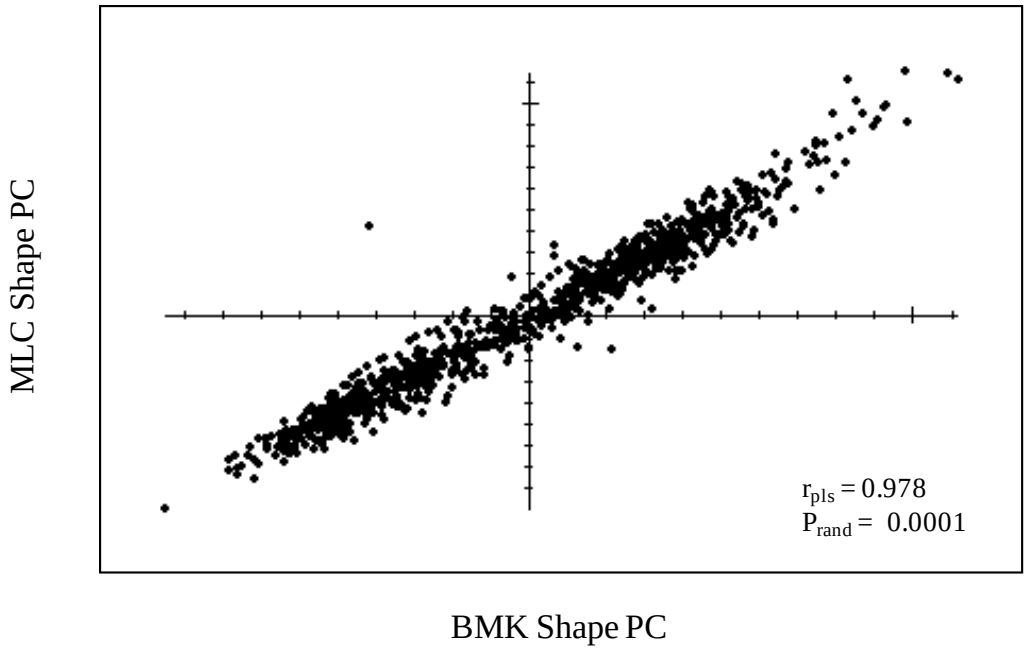

Figure E.3 Plot of first 2B-PLS dimension illustrating a strong relationship between 935 White Sands pupfish sampled by Brandon Kowalski (BMK) and Michael Collyer (MLC). Significance was determined by performing 9999 random permutation procedures. The proportion of observations greater or equal to the observed value was considered the significance level.

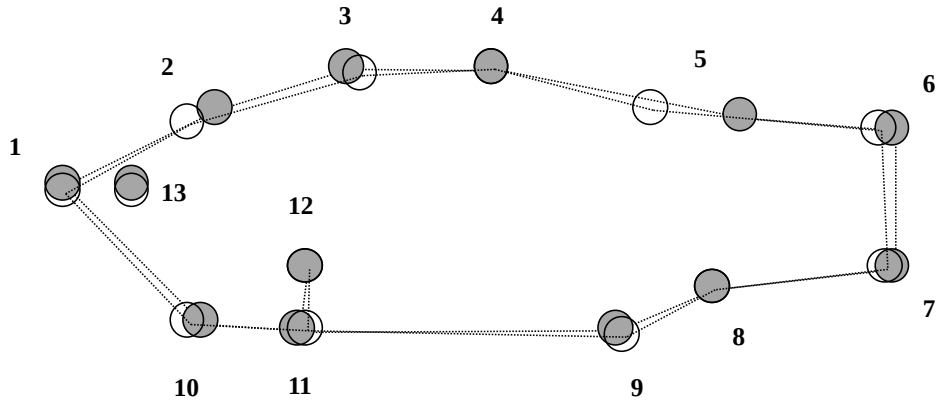

Figure E.4 Consensus shapes depicting measurement differences of 13 anatomical landmarks placed on 935 White Sands pupfish independently by measurers Brandon Kowalski (unfilled) and Michael Collyer (filled). Each consensus shape represent fish collected in 2001 and 2002 (N= 935 for each measurer; Collyer et al. 2005, 2007). The largest observed deviations occurred at landmarks 2, 3, 5, and 10.

APPENDIX F. ALLOMETRIC RELATIONSHIPS AMONG COLLECTED WHITE SANDS PUPFISH POPULATIONS

Although shape variables are constrained by Kendall's definition of shape (mathematically holding size constant), allometric growth will result in covariation between shape and size variation (Chapter 2; Klingenberg 1996). Thus, perceived differences in shape may be misleading in the presence of allometry. This section summarizes the heterogeneity of allometric relationships among all collected White Sands pupfish samples from years 2001, 2002, and 2006; including wild habitats and experimental ponds (N =2012).

A multivariate analysis of covariance (MANCOVA) was performed for the independent variables including sex, pond, year, logCS, and all interactions to test for the covariation of shape and size. Significant allometric relationships were detected, corroborating our decisions for grouping by sex and incorporating size covariation within the models conducted in Chapter 2 (Table F.1).

Biplots were produced to graphically illustrate these relationships. Linear relationships for allometry are shown for each population by year below (Figure F.1 - Figure F.7). Each linear relationship represents the first relative warp score (RW1), corresponding to 25.2% and 26.8% of the total explained body shape variation for pooled male and female populations, respectively. The first relative warps score separates fish in terms of body depth, eye position, and snout orientation/ dorsal arching (see Chapter 2).

Correlation analyses were conducted by sex but are shown in the same figure- males (filled) and females (unfilled). The slope of the regression line illustrates the strength of allometry (e.g., steeper lines indicate greater size by shape change). Negative

or positive relationships are meaningless due to arbitrary rotations within a principal component analysis. The correlation coefficients (r; which is a measure of the strength of the linear relationship), and significance (testing the null hypothesis of the independence of *y* and *x*) was calculated using univariate Pearson correlation analyses (Rencher 2000).

Table F.1 Multivariate analysis of covariance analyzing the phenotypic effects of sex, pond, year, logarithmic centroid size (logCS), and all interactions. All terms were significant except the interaction sex x pond x year x logCS.

Source	Pillai's Trace	F	Num df	Den df	P	Significance
SEX	0.864	305.51	22	1055	<0.0001	***
POND	1.884	14.87	176	8496	<0.0001	***
YEAR	0.422	35.06	22	1055	<0.0001	***
LOGCS	0.648	88.12	22	1055	<0.0001	***
SEX:POND	0.617	4.04	176	8496	<0.0001	***
SEX:YEAR	0.115	6.21	22	1055	<0.0001	***
POND:YEAR	0.449	39.01	22	1055	<0.0001	***
SEX:LOGCS	0.219	13.48	22	1055	<0.0001	***
POND:LOGCS	0.327	2.06	176	8496	<0.0001	***
YEAR:LOGCS	0.073	3.76	22	1055	<0.0001	***
SEX:POND:YEAR	0.064	3.27	22	1055	<0.0001	***
SEX:POND:LOGCS	0.217	1.35	176	8496	0.0016	**
SEX:YEAR:LOGCS	0.035	1.76	22	1055	0.0168	*
POND:YEAR:LOGCS	0.044	2.20	22	1055	0.0012	**
SEX:POND:YEAR:LOGCS	0.027	1.34	22	1055	0.1364	NS

Models were considered significant at the 95% confidence level (i.e., $\alpha = 0.05$). Significance is denoted by asterisks (coded as: 0 '***', 0.001 '**', 0.01 '*') or NS (Non-significant). Significance associated with specimen size (logCS) indicates that shape change is associated with fish growth, and that significant interactions among main effects associated with logCS have very different allometric patterns among groups.

Size by Shape Relationships among White Sands Pupfish Populations

Year 2001

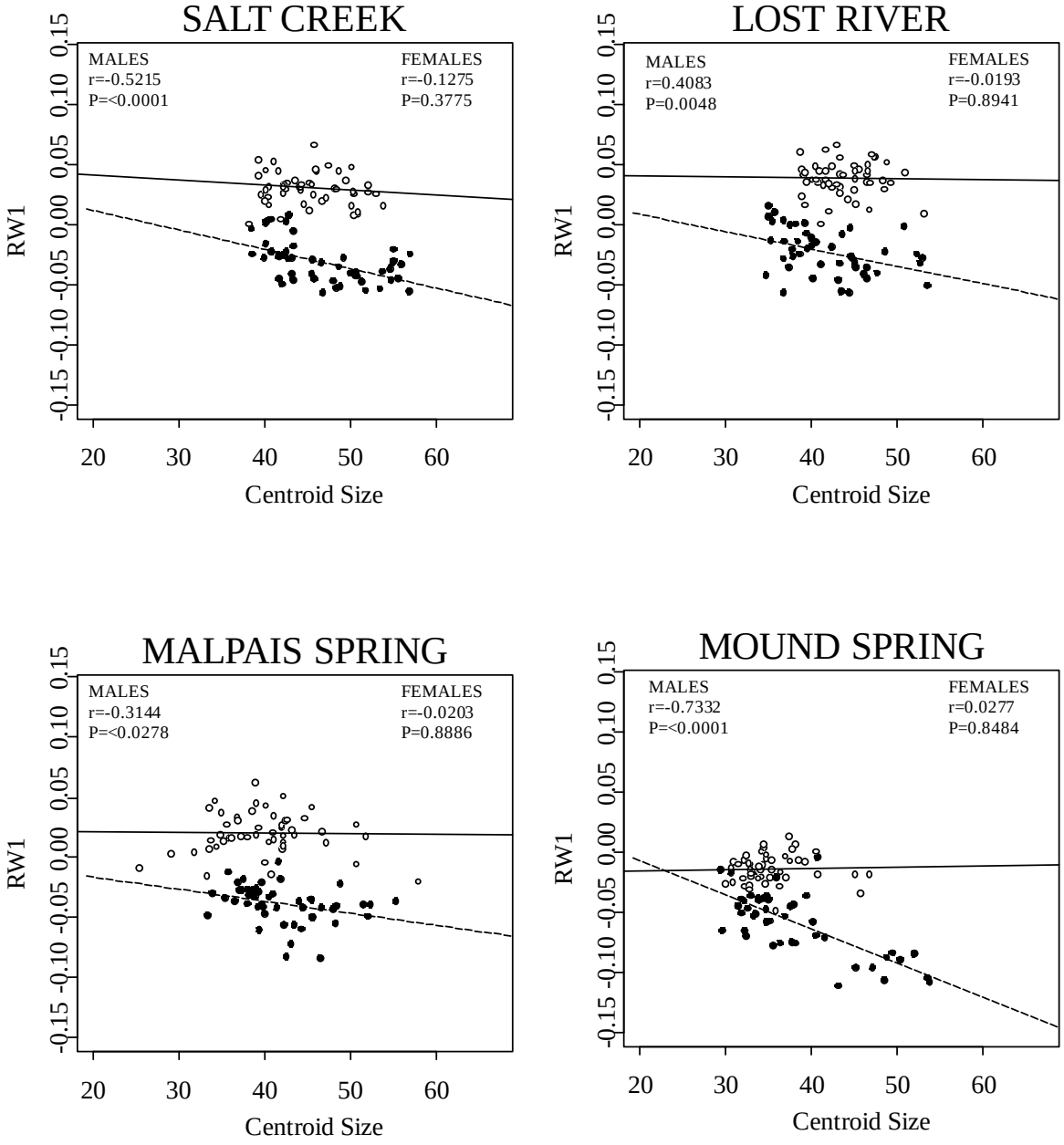

Figure F.1 Biplot of the first relative warps score of shape variation against centroid size for year 2001 Salt Creek, Lost River, Malpais Spring, and Mound Spring populations-males (filled) and females (unfilled). Data were realigned using tpsRelw v. 1.46 and rotated together using PCA procedures in R, v. 2.11.1. Correlation coefficients, r, and significance were calculated with univariate Pearson correlation analyses.

Year 2002

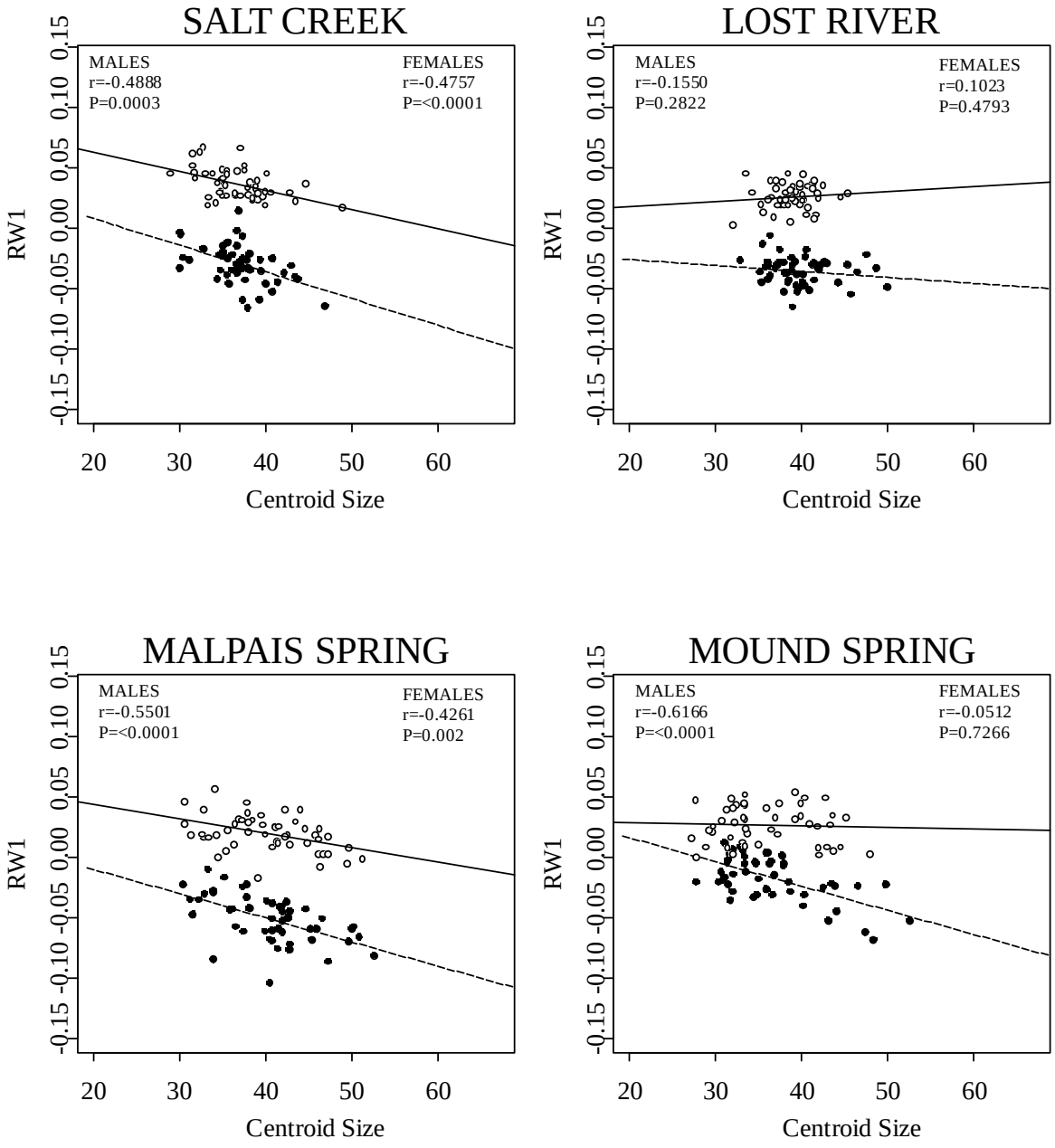

Figure F.2 Biplot of the first relative warps score of shape variation against centroid size for year 2002 Salt Creek, Lost River, Malpais Spring, and Mound Spring populations-males (filled) and females (unfilled). Data were realigned using tpsRelw v. 1.46 and rotated together using PCA procedures in R, v. 2.11.1. Correlation coefficients, r, and significance were calculated with univariate Pearson correlation analyses.

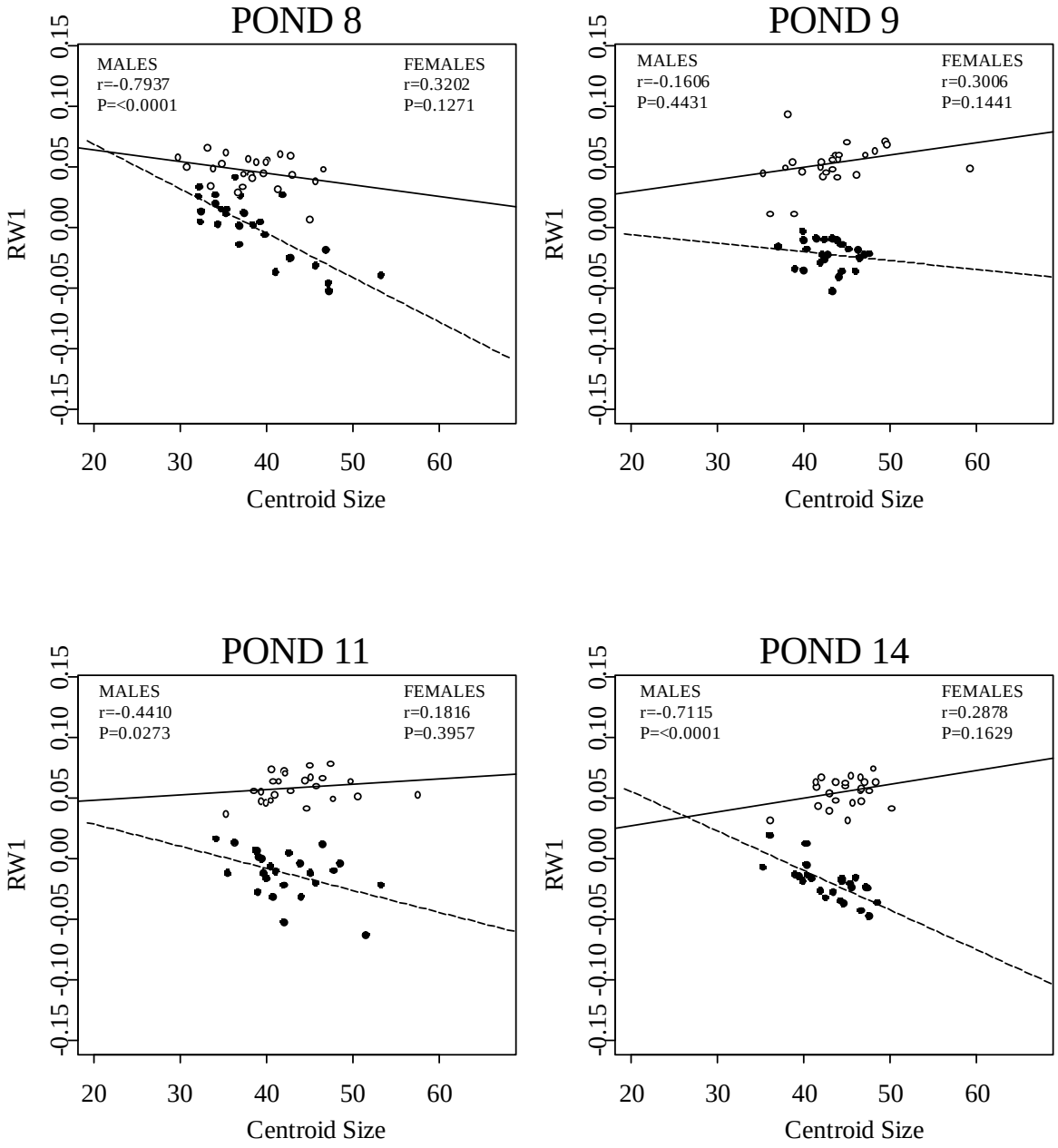

Figure F.3 Biplot of the first relative warps score of shape variation against centroid size for year 2002 pond 8, pond 9, pond 11, and pond 14 populations- males (filled) and females (unfilled). Data were realigned using tpsRelw v. 1.46 and rotated together using PCA procedures in R, v. 2.11.1. Correlation coefficients, r, and significance were calculated with univariate Pearson correlation analyses.

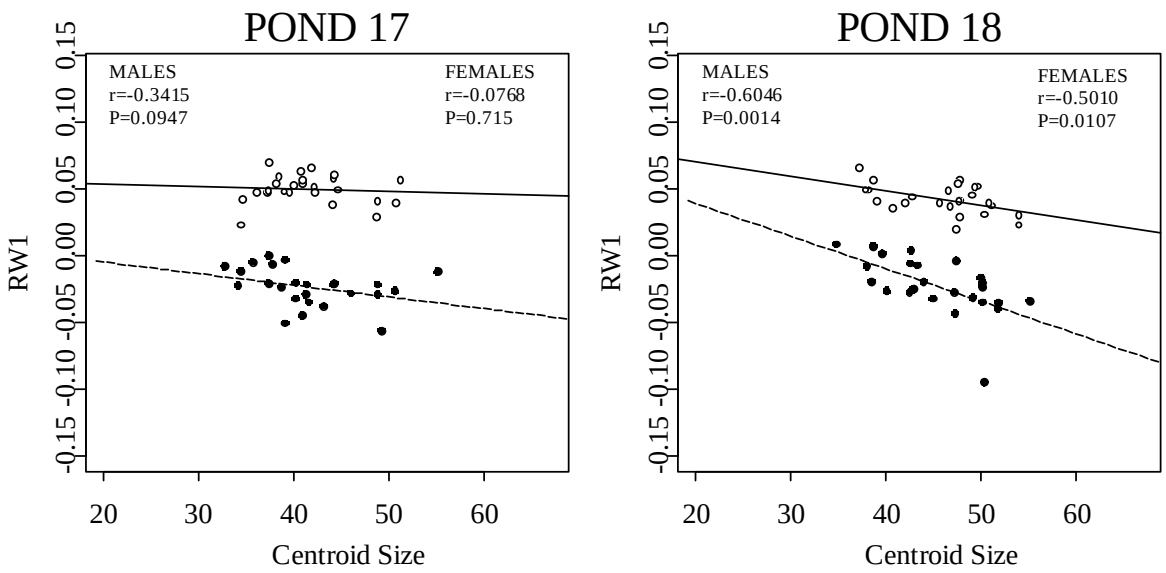

Figure F.4 Biplot of the first relative warps score of shape variation against centroid size for year 2002 pond 17, and pond 18 populations- males (filled) and females (unfilled). Data were realigned using tpsRelw v. 1.46 and rotated together using PCA procedures in R, v. 2.11.1. Correlation coefficients, r, and significance were calculated with univariate Pearson correlation analyses.

Year 2006

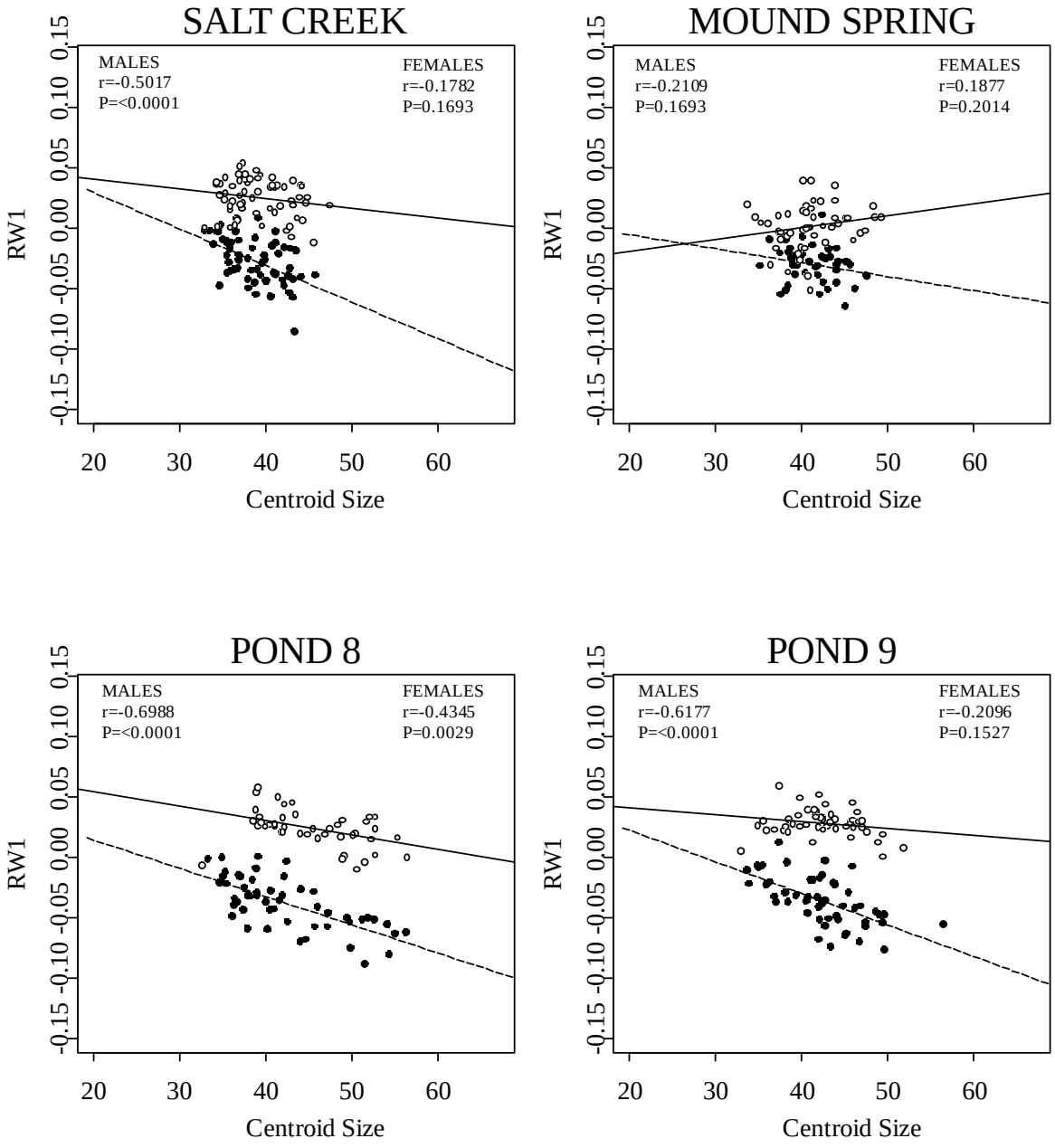

Figure F.5 Biplot of the first relative warps score of shape variation against centroid size for year 2006 Salt Creek, Mound Spring, pond 8, and pond 9 populations- males (filled) and females (unfilled). Data were realigned using tpsRelw v. 1.46 and rotated together using PCA procedures in R, v. 2.11.1. Correlation coefficients, r, and significance were calculated with univariate Pearson correlation analyses.

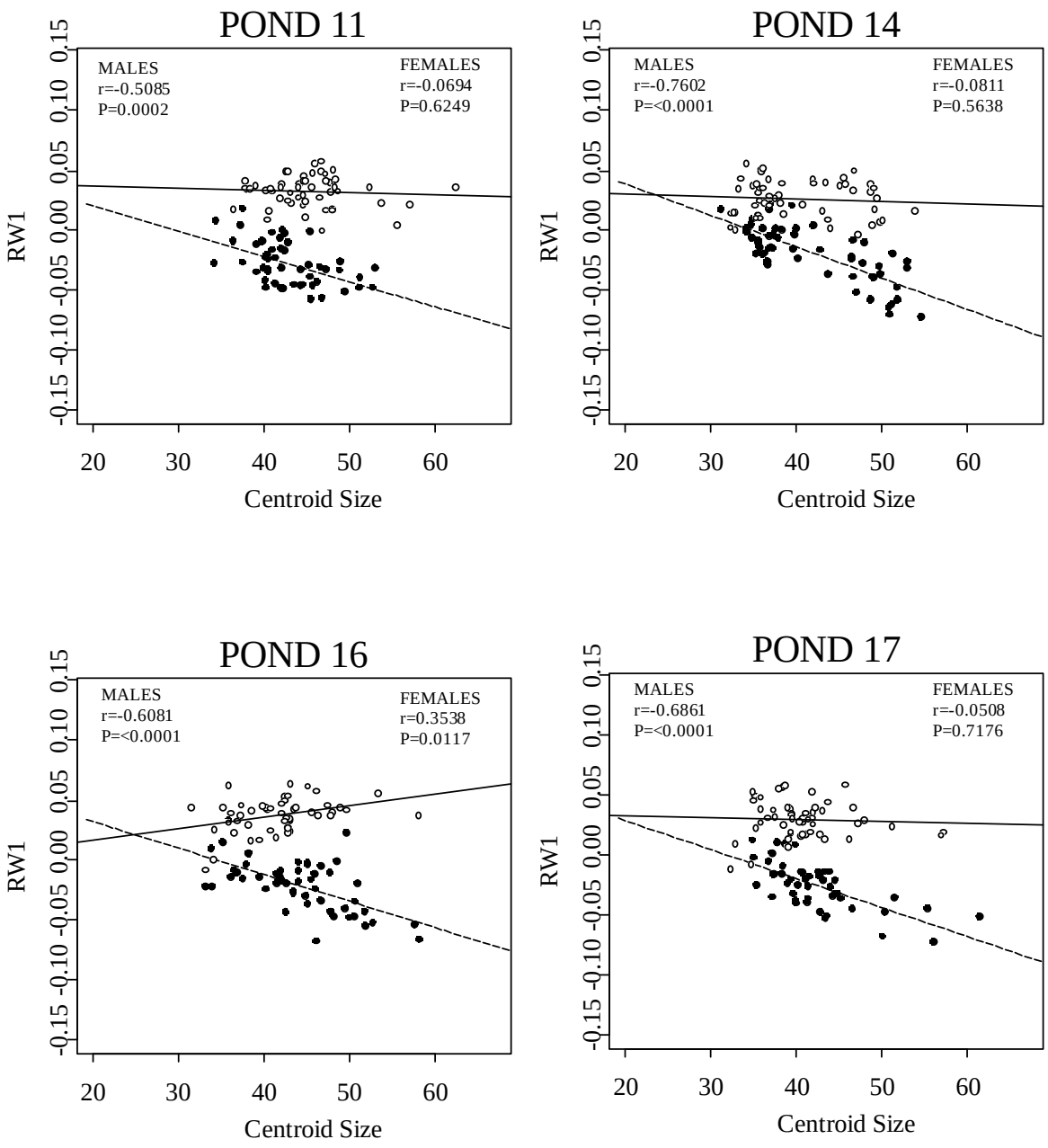

Figure F.6 Biplot of the first relative warps score of shape variation against centroid size for year 2006 pond 11, pond 14, pond 16, and pond 17 populations- males (filled) and females (unfilled). Data were realigned using tpsRelw v. 1.46 and rotated together using PCA procedures in R, v. 2.11.1. Correlation coefficients, r, and significance were calculated with univariate Pearson correlation analyses.

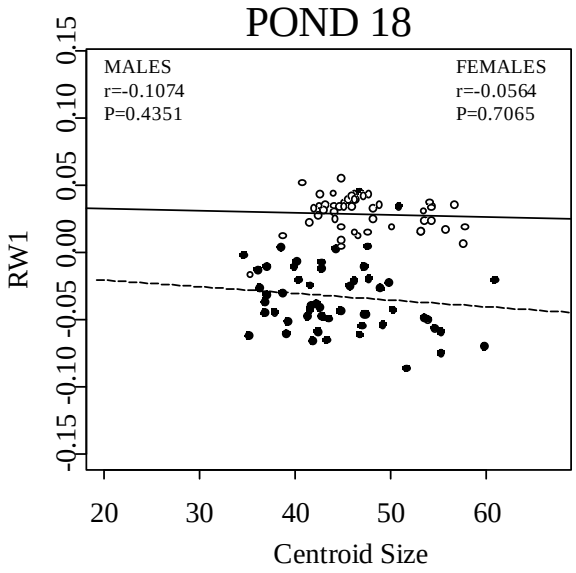

Figure F.7 Biplot of the first relative warps score of shape variation against centroid size for year 2006 pond 18 population- males (filled) and females (unfilled). Data were realigned using tpsRelw v. 1.46 and rotated together using PCA procedures in R, v. 2.11.1. Correlation coefficients, r, and significance were calculated with univariate Pearson correlation analyses.

APPENDIX G. PROBABILITIES OF RANDOM DISTANCES GENERATED IN CHAPTER 2

This section summarizes the probabilities of random distances generated from the performed randomized residual permutation procedure in Chapter 2 (Also see Appendix A). Shape differences among groups were quantified as the Euclidean distance ($D_E = (\Delta\overline{\mathbf{Y}}_i \Delta\overline{\mathbf{Y}}_i^t)^{1/2}$) between population least-squares means after accounting for a common size covariation. Randomized residual permutation procedures were performed using two linear models: 1) a full model that included size covariation (logCS), population, and year effects (including population by year interactions), and 2) a nested reduced model that included only size and year effects. Permutation procedures were performed 10,000 times (including observed values) to generate distributions of pairwise distances (D_E) for determining significance. The proportion of random distances greater or equal to the observed values was treated as the significance of the observed value (Adams and Rohlf 2000; Collyer and Adams 2007). All statistical analyses and graphics were produced in the statistical program R, v. 2.11.1 (available at: http:/www.r-project.org; R Development Core Team 2009).

Table G.1 Probabilities of random Euclidean inter-population distances greater or equal to the observed values in Chapter 2.

	P-8	P-9	P-11	P-14	P-16	P-17	P-18	MO-2006	SC-2006	MO-2001	SC-2001
P-8		0.3003	**0.0005**	**0.0001**	**0.0003**	**0.0007**	**0.0048**	**0.0001**	**0.0001**		0.5688
P-9	**0.0125**		**0.0027**	**0.0001**	**0.0007**	**0.0004**	**0.0210**	**0.0001**	**0.0001**		0.8172
P-11	**0.0005**	0.1493		**0.0002**	**0.0273**	**0.0008**	**0.0005**	**0.0001**	**0.0001**		0.7139
P-14	0.3327	**0.0003**	**0.0293**		0.4835	**0.0158**	**0.0016**	**0.0001**	**0.0001**		0.2569
P-16	**0.0001**	**0.0001**	**0.0043**	**0.0021**		**0.0441**	**0.0004**	**0.0001**	**0.0001**		0.2536
P-17	0.1173	0.0577	0.2767	**0.0051**	**0.0020**		**0.0020**	**0.0001**	**0.0001**		0.3604
P-18	**0.0003**	**0.0215**	**0.0377**	0.1796	**0.0333**	0.4335		**0.0004**	**0.0001**		0.0810
MO-2006	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**		**0.0001**	**0.0001**	0.4132
SC-2006	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**			0.5991
MO-2001								0.6835			**0.0001**
SC-2001	0.0502	0.1520	0.7582	**0.0051**	0.1007	0.4357	0.9134		**0.0001**	**0.0001**	

Values above diagonal are for males, values below are for females. Shading separates allochronic comparisons (gray) from synchronic comparisons *sensu* Hendry and Kinnison (1999). Aberrant comparisons were removed for clarity. Probabilities were quantified by performing a randomized residual permutation procedure (Adams and Collyer 2009) on data from Chapter 2. The proportion of randomly generated distances from 10,000 random pairwise Euclidean distances, including the observed values, greater than or equal to the observed distance was treated as the significance level of the observed value. All significant values (experiment-wise $\alpha < 0.05$) from permutation tests are in bold font.

APPENDIX H. CALCULATED RATES OF DIVERGENCE AMONG EXPERIMENTAL WHITE SANDS PUPFISH POPULATIONS

In this section, the observed phenotypic divergence among experimental White Sands pupfish populations (reported in Chapter 2) was converted to Haldanes and compared to reported divergences within similar studies.

By definition, a Haldane is a standardized metric for the rate of evolution (standard deviations per generation; Gingerich 1993; Hendry and Kinnison 1999; Gingerich 2001). Haldanes were calculated by quantifying vector distances following Adams and Collyer (2009) as:

$$h = \sqrt{D_{mahal}}/g, \qquad \text{Eq. H.1}$$

where, D_{mahal} is the Mahalanobis distance representing the amount of phenotypic change between group means sampled at known time points (e.g., Mahalanobis distance for group means between 2001 and 2006), and g is the number of generations separating the samples (years divided by generation length; D C Adams, Pers. Comm.; Kinnison and Hendry 2001). Haldanes were estimated assuming that one to two generations occurred per year in the experimental White Sands pupfish populations (Collyer et al. 2005). Mahalanobis distances were found using the statistical program R, v. 2.11.1 (http:/www.r-project.org; R Development Core Team 2009).

Overall divergence for experimental 2006 specimens was compared to the derived founding SC-2006 sample (synchronic design sensu Hendry and Kinnison 1999). Notably, synchronic designs are appropriate for inferring rates of divergence, because, by definition, synchronic designs compare shape differences among extant populations from a common

origin. All allochronic comparisons were removed from analysis due to inherent experimental limitations (see Chapter 2).

Because evolutionary rates are negatively correlated with the length of time over which they are measured (i.e., a phenomenon called self-correlation; Gould 1984), estimated Haldane rates within this study were compared to studies reporting Haldanes for organisms within a similar time period.

Following 5 years of monitoring, sampled male and female populations ranged in Haldanes from 0.234 to 0.253 (mean ± 0.245 SE = ± 0.0009) and 0.238 to 0.262 (mean ± 0.254 SE = ± 0.0025) after 5 generations, respectively (Table H.1). In comparison, male and female Haldane rates ranged from 0.468 to 0.506 (mean ± 0.490 SE = ± 0.001) and 0.477 to 0.524 (mean ± 0.508 SE = ± 0.002) after 10 generations (Table H.1).

Table H.1 Observed rates of divergence among experimental White Sands Pupfish populations over 5 years.

	Males				Females			
Pond	D_{mahal}	Haldanes			D_{mahal}	Haldanes		
P-8	6.008	0.245	-	0.490	6.569	0.256	-	0.512
P-9	5.758	0.239	-	0.479	6.830	0.261	-	0.522
P-11	6.240	0.249	-	0.499	6.873	0.262	-	0.524
P-14	6.160	0.248	-	0.496	6.854	0.261	-	0.523
P-16	6.110	0.247	-	0.494	6.189	0.248	-	0.497
P-17	5.493	0.234	-	0.468	6.441	0.253	-	0.507
P-18	6.403	0.253	-	0.506	5.699	0.238	-	0.477
Avg.	*6.024*	*0.245*		*0.490*	*6.493*	*0.254*		*0.508*

Rates of divergence for ponds were calculated from the 2006 Salt Creek Sample (Synchronic design sensu; Hendry and Kinnison 1999). Haldanes were calculated as the square root of Mahalanobis distances (D_{mahal}) between mean groups, divided by the number of generations. Haldanes are reported as a range from 1 and 2 generations per year (i.e. 5-10 generations).

Compared to similar studies (reviewed in Hendry and Kinnison 1999), the observed Haldanes were similar to those reported for Trinidadian guppies (*Poecilia reticulate*, Haldanes: 0.267 – 0.742, Years: 1.8, Generations: 3, Endler 1980) and Galapagos Finches (*Geospiza fortis*, Haldanes: -0.372 – 7.09, Years: 2-3, Generations: 2-3, Grant and Grant 1995), and much higher than the rates reported for Bahamanian lizards (*Anolis sp.*, Haldanes: 0.004 – 0.105, Years: 10-14, Generations: 10-14, Losos et al. 1997).

APPENDIX I. LABORATORY EXPERIMENTS

Evaluating Body Shape Overlap between the White Sands Pupfish and a Surrogate Species, the Sheepshead Minnow (Cyprinodon variegatus)

Formerly described by Miller and Echelle in 1975, the White Sands pupfish (*Cyprinodon tularosa*), Family Cyprinodontidae, is a member the sheepshead minnow (*Cyprinodon variegatus*) complex (Miller and Echelle 1975; Echelle et al. 2005). Sheepshead minnows are the most geographically widespread of the pupfishes and are known for occupying diverse habitat types. Much like White Sands pupfish, physiologically sheepshead minnows are capable of tolerating the most eurythermal and eurysaline conditions of most known fishes. Moreover, sheepshead minnows are morphologically similar (i.e., in a broad sense) to White Sands pupfish, making them ideal surrogates[1].

To investigate suitability of sheepshead minnows as surrogates, body shape was quantified from 90 randomly selected White Sands pupfish from Chapter 2 and 90 sheepshead minnows from two ancillary pupfish projects (i.e., a preservation experiment, sample 14, N = 30; and a swimming performance experiment, N = 60). Sex was combined without distinction among samples, and body shape differences were quantified using the methods described in Appendices A and J. Body shape data from sheepshead and White Sands pupfish were realigned together using tpsRelw v. 1.46 (Rohlf 2008) and statistically analyzed using a Multivariate analysis of covariance (MANCOVA), which accounted for specimen size (logCS; common allometry). MANCOVA revealed significant body shape variation between the two species (Pillai's trace = 0.912, $F_{22, 156} = 74.14$, $P < 0.0001$).

1 Experimental use of Sheepshead minnows was approved by the North Dakota State University IACUC Protocol # A0902

To provide a metric regarding the discrimination between the species effect, a discriminant function analysis (DFA) was performed. A DFA examines the separation between two groups of observations (Timm 2002). DFA are often used to support the identification of distinct species. The assumption is that low rates of misclassification from two populations provide evidence of speciation (Sheets et al. 2006). DFA was performed in MorphoJ v. 1.01C (available at: http://www.flywings.org.uk/MorphoJ_page.htm; Klingenberg 2011) using shape variables from both White Sands pupfish and sheepshead minnows as the dependent variables and SPECIES as the independent variable. In effect, DFA examined how well sheepshead minnows and White Sands pupfish could be classified based on their morphology.

The DFA (cross-validation) classified 167 (92.7%, n = 180) fish appropriately. Minimal overlapping in cross-validation discriminant scores occurred between species (Figure I.2). Based on 9999 iterations, DFA also indicated clear morphological differences between species (D_{mahal}= 3.392, T^2 = 511.29, P <0.0001; Figure I.1).

In conclusion, using both methods, distinct body shapes between White Sands pupfish and sheepshead minnows were evident, corroborating the species designation (Miller and Echelle 1975; Echelle et al. 2005). Nonetheless, despite these findings, both sheepshead minnows and White Sands pupfish appear to occupy similar locations in morphospace (Figure I.1), providing enough overlap and variation to warrant the use of sheepshead minnows as an appropriate surrogate for the White Sands pupfish.

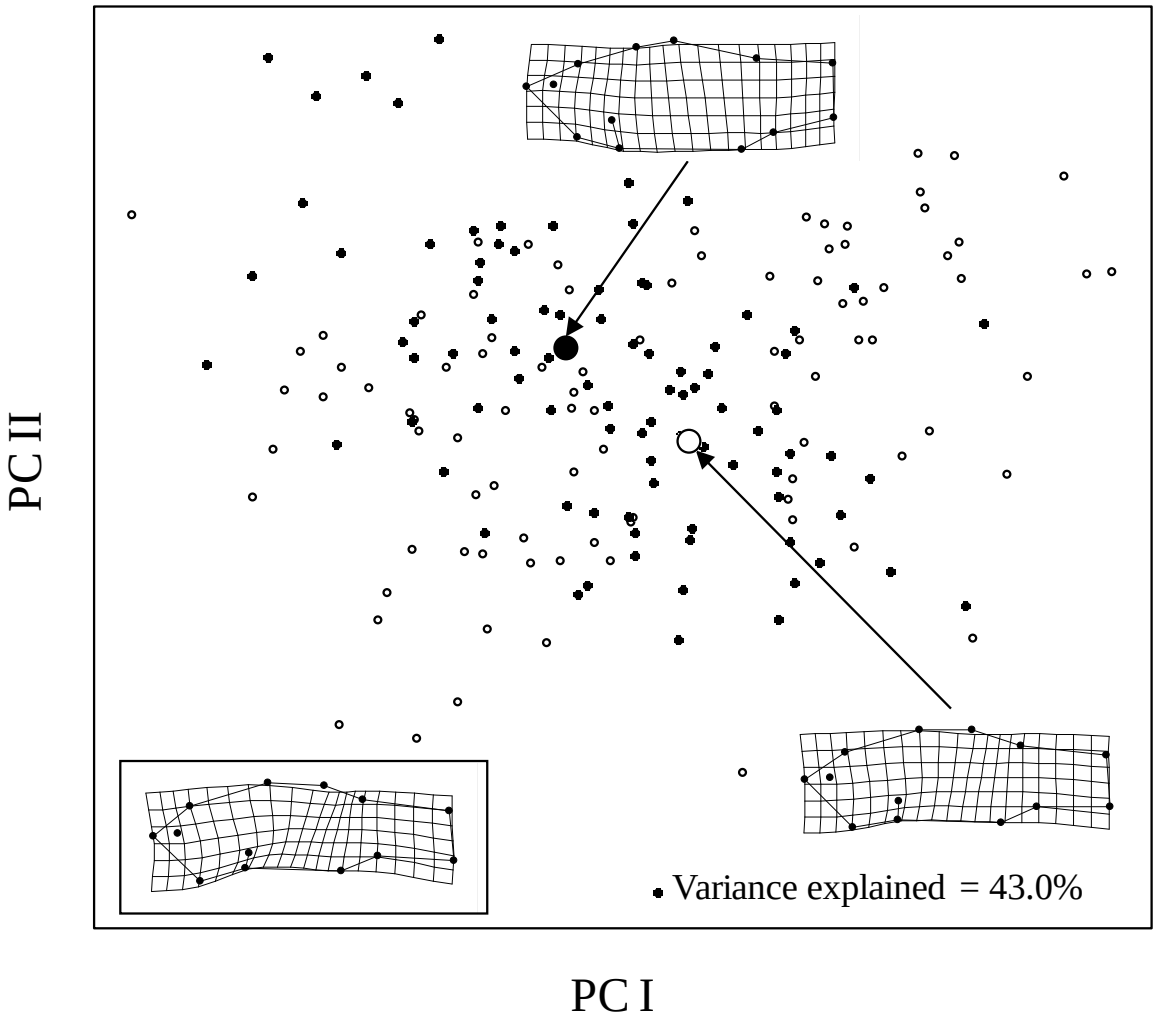

Figure I.1 Principal component plot demonstrating overlapping body shape variation between White Sands pupfish (N=90; Black) and sheepshead minnows (N=90; White). The bars represent 95% confidence intervals from the mean. Thin-plate spline deformation grids for representative samples are shown. The arrowed deformations represent mean body shapes among species. The boxed deformation represents mean shape change between the sheepshead minnow and White Sands pupfish. Deformations correspond to the first two principal components of relative warps analysis (43.0% of the total shape variation). Deformations were accentuated by a factor of 3 to amplify shape change and facilitate a visual understanding of shape change among groups.

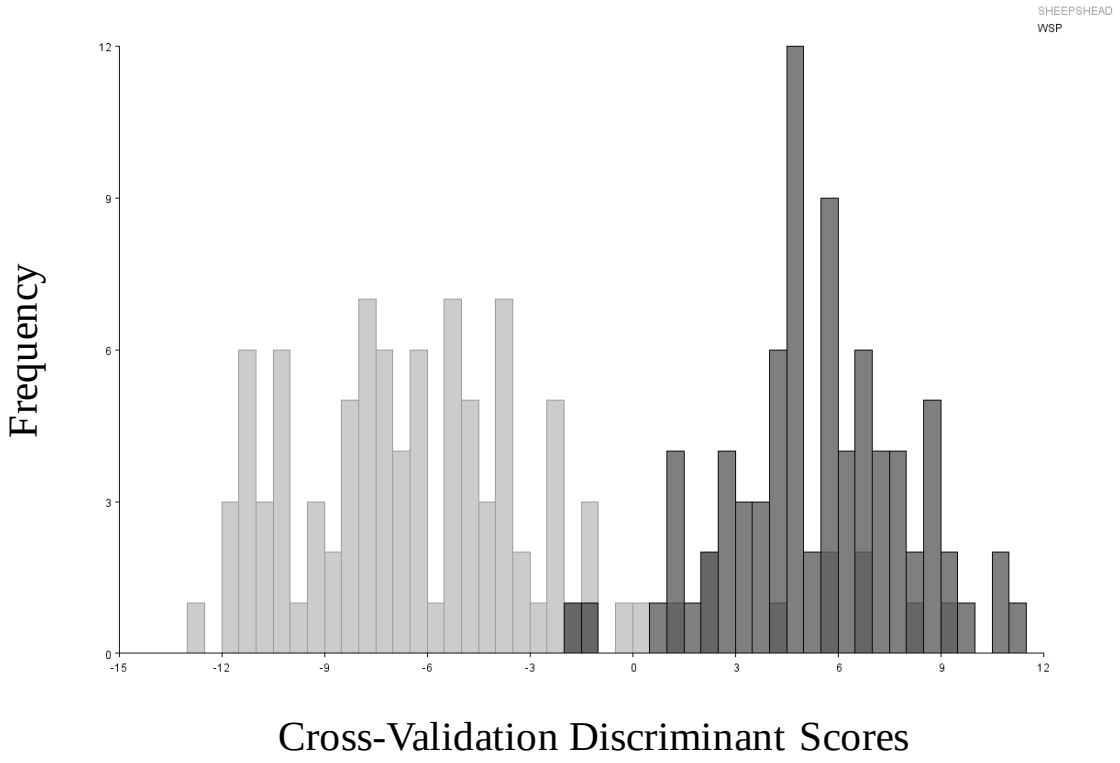

Figure I.2 Results from discriminant function analysis (DFA) examining the separation of body shape data between White Sands pupfish (dark gray) and Sheepshead Minnows (light gray). DFA cross-validation correctly classified 92.7% of individuals based on body morphology.

Potential Artifacts of Geometric Morphometric Analyses: Effects of Formalin Preservation using Sheepshead Minnows

Introduction

Preservation can induce changes in fish tissue and structure (Shields and Carlson 1996). One possible hazard of using geometric morphometric techniques on museum or archived specimen is that fixation and preservation may deform bodies or body parts. This artificial alteration may confound analyses, especially when comparing live fish to museum specimens.

Various preservation effects have been formerly described for fish (Shetter 1936; Parker 1963; Stobo 1972; Shields and Carlson 1996; Sagnes 1997); however, the effects of preservatives on fish morphometrics have been shown to be equivocal or unpredictable (Sagnes 1997). Formalin, the preferred preservative in many studies, effectively replaces water in tissues soon after the fish dies, permitting tissues to retain their rigidity. Thus, the term, 'fixed' or 'fixation', refers to the specimen becoming rigid (Sturgess and Nicola 1975). However, in some cases, formalin has been reported to cause shrinkage of fish (Parker 1963, Jennings 1991, Takizawa et al. 1994, Shields and Carlson 1996); whereas, in other cases, it has been reported to increase fish length (Billy 1982). Variances related to the type of preservative, duration of preservation, origin of species (marine vs. freshwater fish), species, and life stages have shown to have confounding influences (Sagnes 1997), albeit no studies have clearly quantified preservation effects on body shape using landmark based geometric morphometric techniques.

In a previous study, Collyer et al. (2005, 2007) examined body shape variation among several White Sands pupfish populations occupying ecologically dissimilar

environments. Body shape variation was quantified using landmark-based geometric morphometric techniques. Images of fish from 2001 and 2002 were immediately captured after fish were euthanized with tricaine methanesulfonate (Tricaine-S® MS-222; Western Chemical, Inc., Ferndale, WA; Summerfelt and Smith 1990). For this disquisition, images of White Sands pupfish collected in 2006 were captured from formalin-preserved fish approximately 4 years after collection. In view of unpredictability and lack of information among geomorphometric studies, the aim of this study was to investigate whether significant body shape changes would accrue in euryhaline pupfish due to preservation in 10% formalin solution. The results from this study will prove important for geometric morphometric studies when comparing live fish to museum specimens.

Methods

In this study two-hundred sheepshead minnows were obtained from a commercial supplier (Aquatic Biosystems Inc., Ft. Collins, Colorado, U.S.A.) in April 2010. Fish age varied from approximately 105 d to 135 d. These fish were reported to be most likely derived from two wild populations three years prior to this study; New Port News, VA and a laboratory near Houston, TX. At the facility, sheepshead minnow populations were reared together under temperatures at 23°C, Salinities at 25 ppt, Total Alkalinities at 120 mg/L, and pH at 7.59. The gene pool is now considered to be homogenous between these two populations (S Kellman, Pers. Comm.).

A total of 30 sheepshead minnows (14.38-31.17 mm SL, sex unknown) were used in this study. Sheepshead minnows were processed according to standard preservation protocols (Hubbs & Lagler, 2004). Fish were euthanized in approximately 500 mg/L tricaine methanesulfonate (Tricaine-S® MS-222; Western Chemical, Inc., Ferndale, WA;

Summerfelt and Smith 1990; NDSU IACUC # A0902). Specimens were photographed immediately after euthanasia (referred to as day 0), and on a biweekly basis until day 84. All specimens were fixed in a 10% formalin solution (made from distilled water) for 7 days, decanted, transferred to a successive bath of 10% formalin solution for 1 day, decanted, and placed into a final 10% formalin solution for archival storage. Each successive bath was adopted to ensure the homogeneity of concentrations among samples. A single hobo temperature logger (HOBOware®) was used to measure ambient room temperatures every 2 hours. Room temperatures ranged from 19.75 °C to 26.88 °C with an average temperature of 20.95 °C for the duration of the experiment.

To quantify body shape, the left lateral body surface (Kelsch and Shields 1996; Hubbs and Lagler 2007) of White Sands pupfish was analyzed using landmark-based geometric morphometric techniques (See Appendices A and J; Rohlf and Marcus 1993; Adams et al. 2004). Thirteen anatomically homologous landmarks were used for the collection of body shape (Figure A.1, Table A.1; Collyer et al. 2005, 2007). TPS software (available at: http://life.bio.sunysb.edu/morph/) was used for extracting and processing shape data. Images of fish were digitized in tpsDig v. 2.14 (Rohlf 2009a) all by one measurer (BMK).

To ground-truth the effects of preservation on specimens, a repeated measures ANCOVA (Pillai's trace test statistic, type III SS model) was performed to evaluate temporal body shape variation (RW1), while accounting for common allometries. Significance was determined at an alpha level of 0.05 (i.e., 95% confidence level). Shape differences in shape space were depicted through thin-plate spline deformations (Bookstein 1989; Bookstein 1991). Deformations were accentuated by a factor of 3 to amplify shape

change and facilitate a visual understanding of deformation among samples. All Statistical analyses and graphing were conducted using TPS series software (http://life.bio.sunysb.edu/morph/) and the statistical program R, v. 2.11.1 (http:/www.r-project.org; R Development Core Team 2009).

Results

Repeated measures ANCOVA performed on formalin preserved specimen revealed significant variation among biweekly samples ($F_{6, 29} = 4.446$, $P = 0.0003$; Figure I.3); albeit, Mauchly's Tests for sphericity indicated unequal variance between levels of repeated measures ($W = 0.166$, $P<0.0001$). Investigations into the preservation effect (residuals) suggest that day 0 specimen exhibited considerably more variation than all subsequent biweekly samples, which likely contributed to the observed sphericity significance. Interestingly, day 14 - 84 samples appear to stabilize over time, indicating that most significant shape change maybe occurring within the first 14 days (Figure I.4). Pairwise tests revealed that day 0 (non-preserved fish) significantly differed from all subsequent biweekly samples ($P \leq 0.01$), with the exception of day 56 ($P = 0.064$).

Discussion

Overall, body shape appears to change in response to formalin (Figure I.3). Relative to day 0, noticeable expansion in the head, body, and caudal peduncle region were observed among all subsequently sampled specimens (Figure I.3). In addition, all subsequently sampled specimens exhibited dorsally-anterior eye positions, relative to day 0. Notably, inter-sample distinctions among thin-plate spline deformations among biweekly samples between days 14 -84 appear to be due to measurement error in the

placement of landmarks 2 and 3. These distinctions were also observed when investigating the repeatability of shape measurements (see Appendix E).

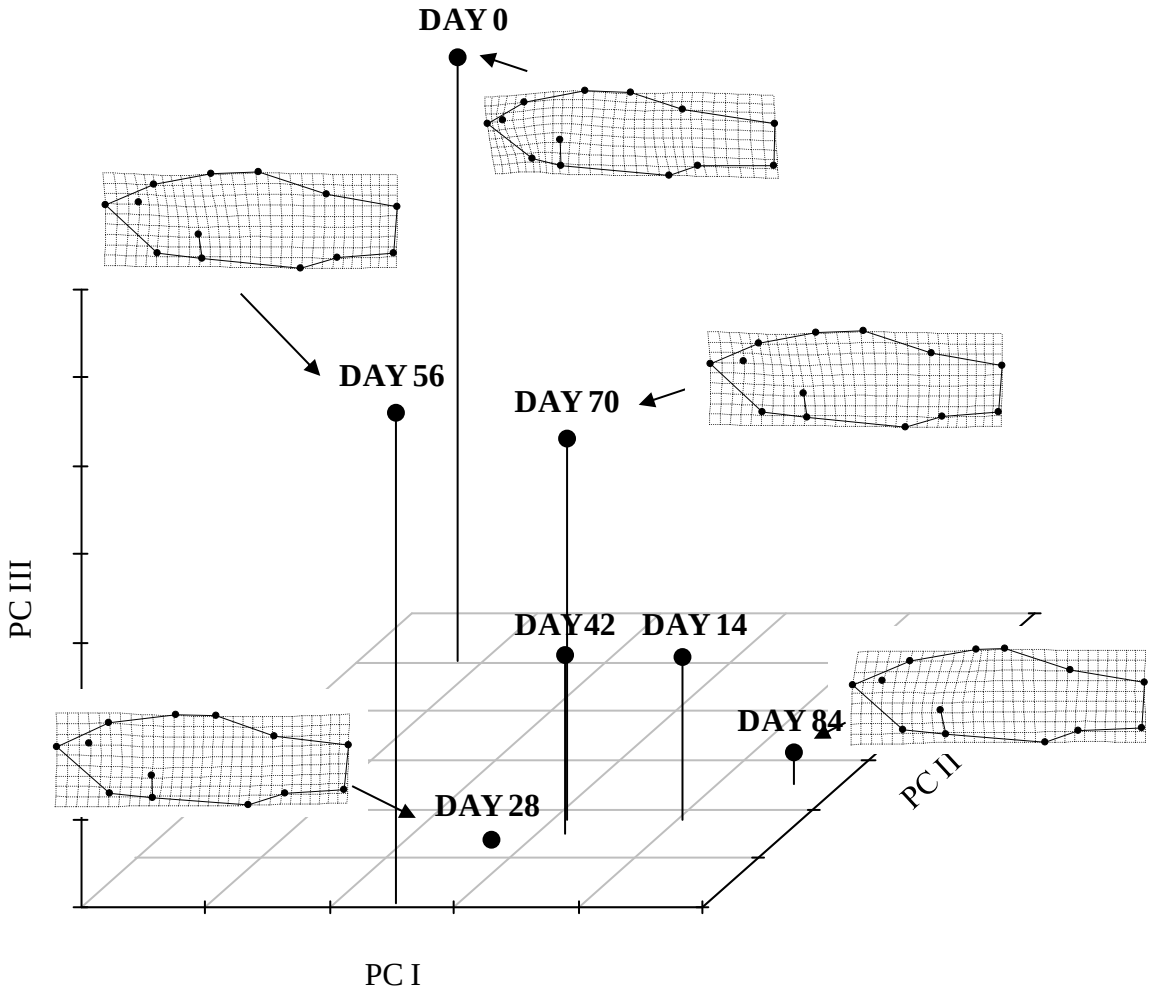

Figure I.3 Principal component plot of thirteen landmark configuration shape variation among recently euthanized and preserved sheepshead minnows sampled biweekly (days 0-84). Principle components (PCs) were calculated for shape residuals after regressing shape against logCS. PC plot corresponds to 53.3% of shape variation. Rotations are arbitrary. Thin-plate spline deformation grids for representative samples are shown. Deformations were accentuated by a factor of 3 to amplify shape change and facilitate a visual understanding of shape change among groups.

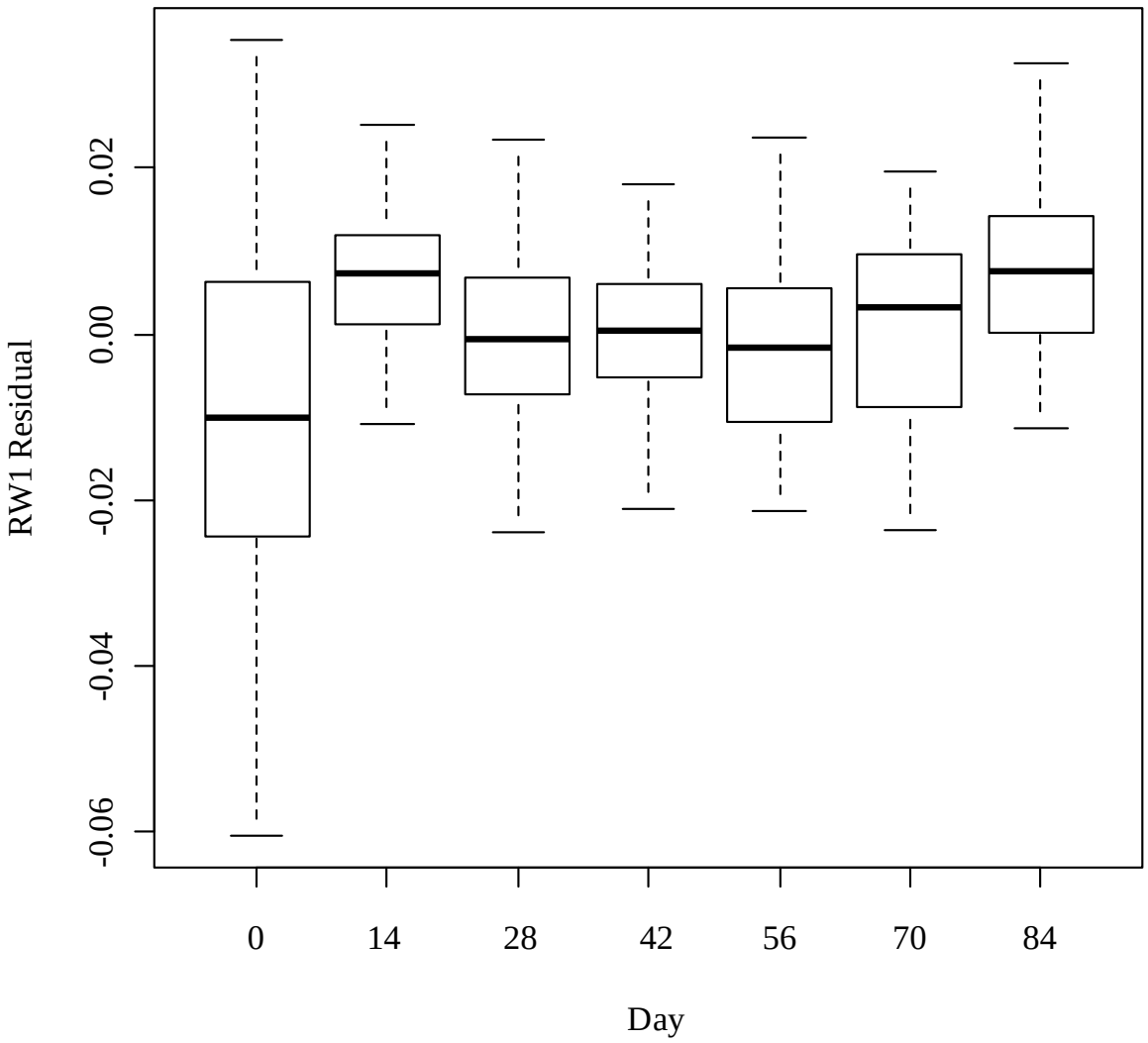

Figure I.4 Preservation effect among sheepshead minnows sampled biweekly. Box plots show the range, 25th percentile, median, and 75th percentile of the first relative warp score residuals. Day 0 illustrates greater variance than the following samples; albeit, variance appears to stabilize immediately within the first 14 days, suggesting a significant preservation effect occurring within the first 14 days.

Investigating Functional Body Shape Advantages to Water Flow using Sheepshead Minnows

Introduction

Water flow is generally believed to be an important factor in fish morphology (e.g., Collyer et al. 2005; Langerhans et al. 2007). Small changes in morphology have been shown to largely affect performance in aquatic environments (Webb 1982, 1984; Weihs and Webb 1984; Webb and Gardiner Fairchild 2001). Therefore, body shape is expected to influence swimming performance in fishes, which is in turn expected to influence components of fitness (Langerhans 2008; 2009, 2010). Annually, the Tularosa Basin goes into a brief wet season, where lentic habitats, such as Salt Creek and Lost River, often encounter flash flooding events. Considering that increased body depths generally confer increased drag, streamlining should reduce drag resistance in such conditions (Webb 1988; Vogel 1994). Mechanistically, it follows, to some degree, that these events may have a selective effect on body shape (Collyer et al. 2005).

Methods

To test whether body shape has an effect on swimming performance, 60 sheepshead minnows were obtained from a commercial supplier (Aquatic Biosystems Inc., Ft. Collins, Colorado, U.S.A.) and used in this study. Fish were reported to be most likely derived from two wild populations three years prior to this study; New Port News, VA and a laboratory near Houston, TX. At the facility, sheepshead minnow populations were mixed and reared under temperatures at 23°C, Salinities at 25 ppt, Total Alkalinities at 120 mg/L, and pH at 7.59. Fish were fed *ad libitum* using premium Tetramin® flake food prior to swimming trials (NDSU IACUC # A0902).

Swimming performance was measured by calculating critical swimming speed (U_{crit}). Critical swimming speed is a standard measurement to assess the swimming capabilities of fishes (Plaut 2001). The critical swimming speed of each individual was determined with the formula of Brett (1964),

$$U_{crit} = V_{-1} + \left(V_d \frac{T}{T_1}\right) \quad \text{Eq. I.1}$$

where, U_{crit} is the critical swimming speed (cm/s), V-1 is the highest velocity maintained for the described time period (cm/s), V_d is the velocity increment (cm/s), T is the time elapsed at the final velocity (s), and T_1 is the time increment (s). Because swimming performance typically varies with fish length (Stahlberg and Peckman 1987), critical swimming speed was length-corrected by dividing an individual's SL (mm) by the observed critical swimming speed.

Critical swimming velocity (U_{Crit}) was measured in a Brett-type apparatus (Brett 1964), constructed by BMK (Figure I.5). The chamber consisted of a transparent PVC pipe (1 m x 2.54 cm I.D.) supplied with water by a 1/6 HP centrifugal pump and regulated by a flow meter (1.64 L system). The pump was placed in a small 7.57 L carboy filled with saltwater (25 ppt, Range: 20.2-26.6 ° C). To limit formation of boundary layers, drinking straws (5 mm I.D.; 22.5 cm long) were used to create random turbulence (i.e., statistically uniform turbulence; Brett 1964) within the chamber. Preliminary tests revealed that no threatening boundary layers could be found. Nonetheless, because of the rate of decay, turbulence over the length of the chamber eventually subsided to laminar flow. Water exiting the chamber flowed back into the carboy after flowing through a vertically positioned 90° PVC elbow joint, forming a recirculating closed system. A 65 watt energy-efficient (i.e., low heat output) florescent bulb was used at the end of the chamber to

discourage fish from exiting the chamber, and the midsection of the chamber was wrapped with duct tape to encourage fish to swim in the darkest section of the chamber. Temperatures were measured with a portable instrument (Yellow Springs Instrument-YSI 85®) and maintained using ice made from stock water (Range: 20.5 – 26.6 °C). Temperature was monitored before and after for incorporating covariation. A net was placed at the end of the chamber to catch fatigued or exhausted fish (see Rosenfield et al. 2004).

To begin trials, fish were inserted at the end of the chamber at an initial velocity of 11.0 cm/s. Once an individual began to swim actively, a digital timer was activated. The flow rate was increased by 2.6 cm/s every 180 seconds until the fish was no longer able to actively swim in the chamber (16.21-34.01 cm/s). To distinguish fatigued fish from unmotivated fish, fish that landed in the net were immediately placed back into the chamber and encouraged to swim (Rosenfield et al. 2004). This process continued until three successive trials failed. Digital timers were stopped once the fish hit the net. Moreover, because the elbow joint disrupted the flow, blunt probes were used to discourage fish occupying this section. Digital timers were temporarily stopped until fish were removed from this section.

After swimming trials, fish were anesthetized using a low dose of tricaine methanesulfonate (approximately 50 mg/L Tricaine-S® MS-222; Western Chemical, Inc., Ferndale, WA; Summerfelt and Smith 1990) and digitally photographed (left lateral surface) using a 3.3 megapixel Canon Powershot G1® digital camera. Fish were placed on standard 3 x 5 inch ruled note cards to provide both contrast and scale, and probes were used to flatten the left lateral surface when necessary. Specimens were subsequently

weighed to the nearest 0.0001 g using an analytical balance (Denver Instrument Company, Model: A-250), and standard length measurements were quantified using ImageJ software (available at: http://rsbweb.nih.gov/ij/).

Thirteen homologous anatomical landmarks were used for the collection of body shape (Figure A.1; Table A.1; Collyer et al. 2005, 2007), and analyzed using landmark-based geometric morphometrics (see Appendices A and J; Rohlf and Marcus 1993; Adams et al. 2004).

Swimming performance and body shape were investigated using regression. The first relative warp score (RW1) served as a proxy for body depth. In this study, RW1 corresponded to 33.9% of the total body shape variation captured. Male and female sheepshead minnows were pooled and body shape was regressed against 1) critical swimming speed (U_{Crit}) and 2) length-corrected swimming speed (U_{Crit} / SL). Biplots were then produced to graphically illustrate relationships. The least-squares regression line was provided to visually illustrate the strength of association. The coefficient of determination, R^2, is a measure of the percentage of the total variation in body shape that is explained by the least-squares regression line (Rencher 2000). The null hypothesis of the independence of y (RW1) and x (critical swimming speed / length-corrected critical swimming speed) was tested (i.e., H_o: $\beta_1 = 0$). Significance was determined at an alpha level of 0.05 (i.e., 95% confidence level). All analyses and graphics were performed in the statistical program R, v. 2.11.1 (available at: http:/www.r-project.org; R Development Core Team 2009).

Results

Swimming performance was positively and significantly associated with body shape (U_{Crit}: $R^2 = 0.1529$, $F_{1,58}$, $P = 0.002$; Length-corrected U_{Crit}: $R^2 = 0.1112$, $F_{1,58}$, $P = 0.009$). Linear relationships with 95% confidence intervals are shown in Figure I.6.

Discussion

Significant relationships between body shape and critical swimming speed were found. These results were not surprising because a water flow is known to affect swimming performance (Webb 1982, Langerhans 2008, 2009, 2010). These findings may indicate that water flow may mediate functional body shape adaptations among pupfishes (Collyer et al. 2005); where, an optimal body shape may be more advantageous under specific flow or foraging conditions (Vogel 1994) by means of reduced drag (Weihs and Webb 1983) or improved burst speeds (Langerhans 2009). Additional work is needed to determine the extent of these findings. Regardless, these results provide a step toward understanding functional body shape advantages to water flow within Cyprinodont fishes.

Acknowledgements

I would like to thank Steven Burdick for volunteering with this project. Steven assisted with the construction and standardization of the Brett-type apparatus, and spent many hours running swimming trials. I would also like to thank Dr. Craig Stockwell for his financial support. C. Stockwell purchased the necessary material for building the Brett-type apparatus. Experimental use of Sheepshead minnows was approved by the North Dakota State University IACUC Protocol # A0902.

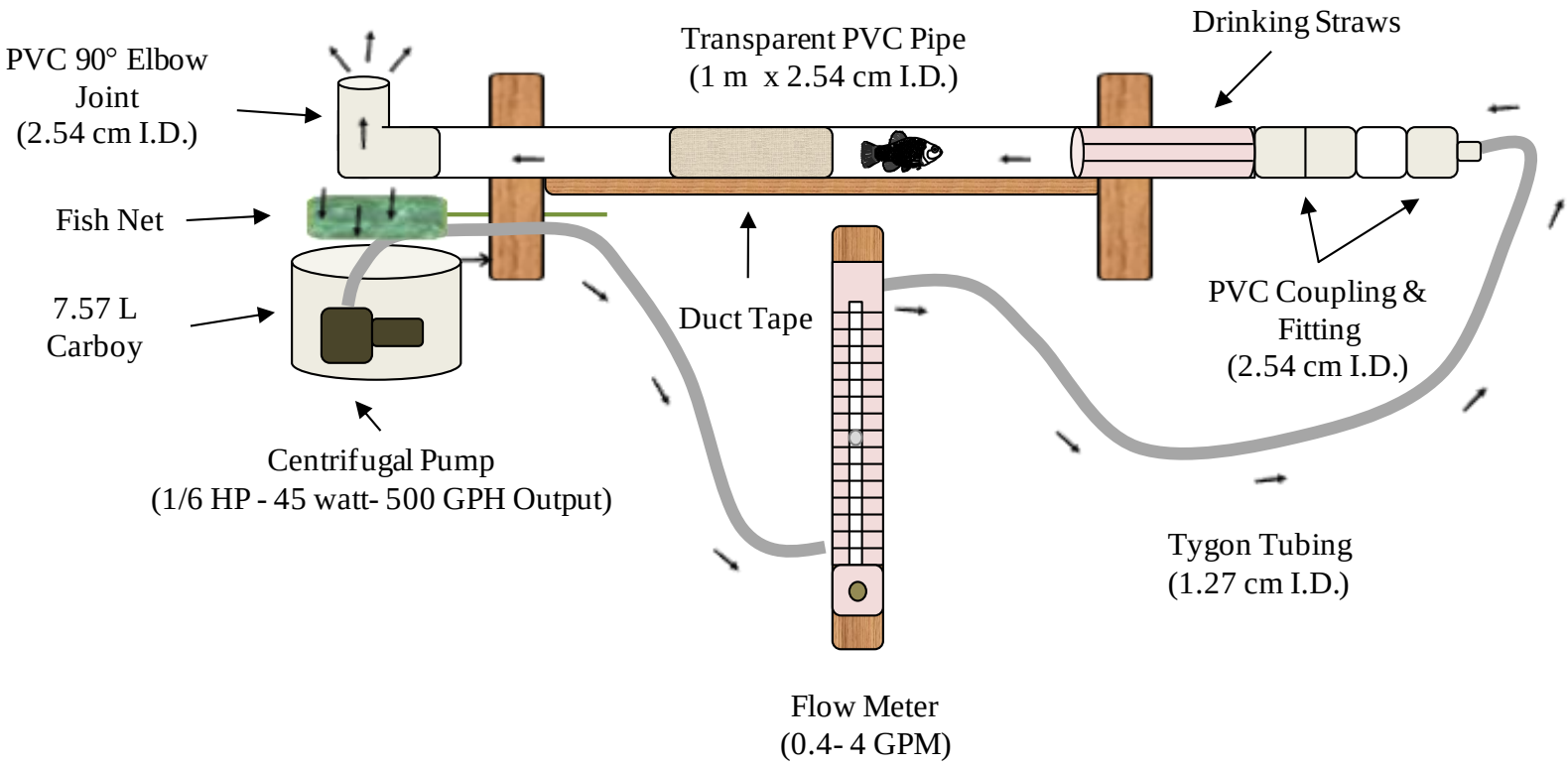

Figure I.5 Diagram of the Brett-type swimming chamber (1.62 L) used to measure swimming performance (light not shown). Swimming performance was quantified by measuring the critical swimming speed of sheepshead minnows. Critical swimming speed was determined and size corrected (critical swimming speed / standard length (mm)) with the formula of Brett (1964). Illustration by Brandon M. Kowalski.

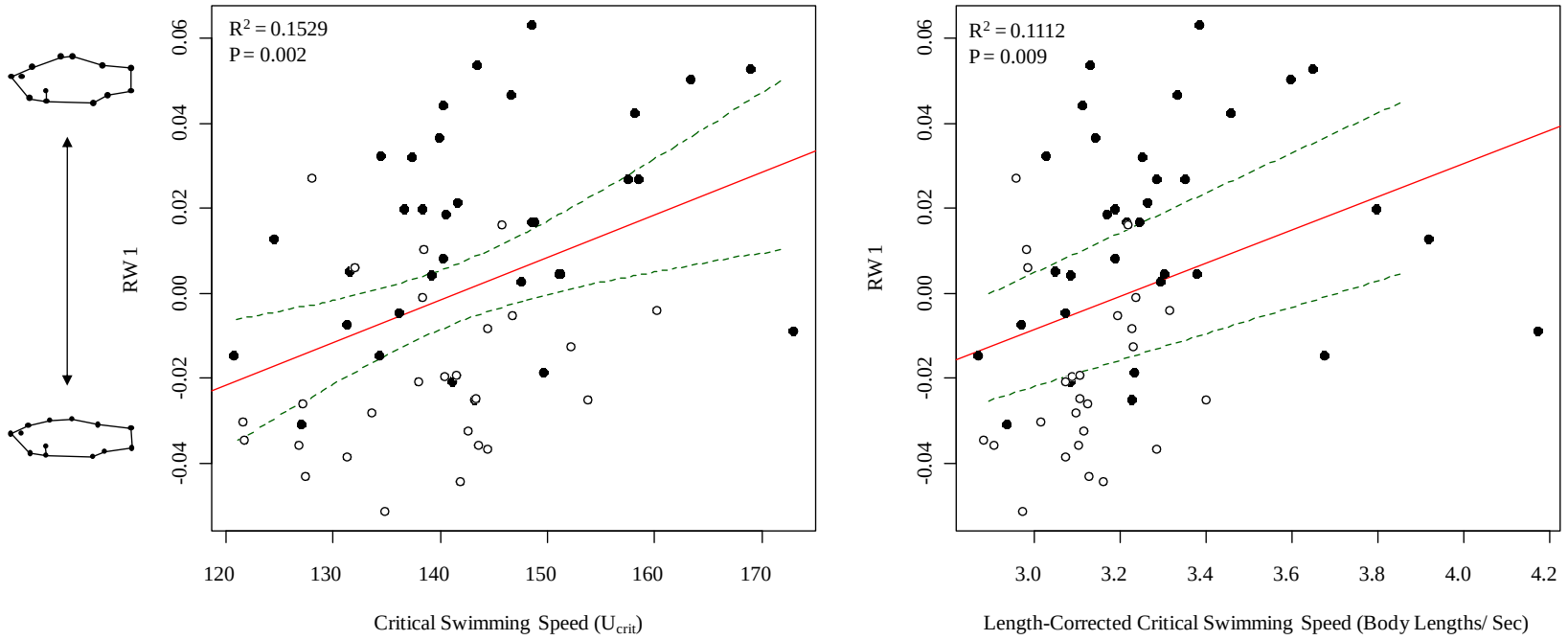

Figure I.6 Biplot of body shape (RW1) against swimming performance for male (filled) and female (unfilled) sheepshead minnows. Solid bar represents the linear fit among groups and hatched bars represent the 95% confidence intervals. RW1 corresponds to 33.9% of the total body shape variation captured. Critical swimming speed (left panel) and length-corrected critical swimming speed (critical swimming speed / standard length (mm); right panel) was measured to quantify swimming performance. Critical swimming speed was determined with the formula of Brett (1964).

APPENDIX J. A PRIMER ON GEOMETRIC MORPHOMETRICS

Introduction

The interpretation of body shape is important to our understanding of many biological phenomena. Shape analysis has been used prominently in studies of phylogenetic relationships, community structure, life histories, animal behavior, ecomorphology, and ecological processes (Richtsmeier et al. 2002; Adams et al. 2004; Zelditch et al. 2004). From the Greek roots, morphometrics stems from the word '*morphe*', translating to the meaning, 'shape'; whereas, the word '*metron*' translates to 'measurement' (Collyer 2003). Geometric morphometrics is thus simply a quantitative way to assess similarities or differences among shapes. My thesis focuses on the use of geometric morphometrics to quantify contemporary body shape evolution and evaluate the repeatability of morphological divergence among experimental and wild habitats of White Sands pupfish (*Cyprinodon tularosa*) in the field. The use of geometric morphometrics is a large component of this disquisition; therefore, the following sections will introduce fundamental concepts of geometric morphometrics.

Landmarks

Shape analyses generally start with the collection of two- or three- dimensional points representing a set of (x, y) or (x, y, z) Cartesian coordinates known as landmarks. Landmarks are selected *a priori* from photographed specimen in a process commonly referred to as digitizing. Landmarks are discrete anatomical loci that must be homologous among all specimens in the study. In general, five criteria should be considered when choosing landmarks. Landmarks should 1) be homologous anatomical loci, 2) be

consistent in location, 3) provide adequate coverage of the shape under consideration, 4) be reliable and repeatable, and 5) lie within the same 2 or 3 dimensional plane (Zelditch et al. 2004).

Bookstein (1991) described landmarks shared by specimen in a study by classifying them into three categories. These categories describe 3 types of landmarks: 1) 'Type I landmarks'- referring to discrete juxtapositions of tissues, 2) 'Type II landmarks'- referring to discrete positions near the tips, maxima/centra, or curvatures of an object (such as the tip of a snout or other morphogenic processes), and 3) 'Type III landmarks'- referring to extremal points (defined by the most extreme distance from another landmark).

Type I landmarks are considered optimal because they are defined by discrete processes, making interpretation easier. Type II landmarks are considered more problematic, because they may lack information from surrounding tissues. Lastly, type III landmarks are considered the least optimal due to the lack of any structural definition. Therefore, landmark selection is cautioned for its effect on biological interpretations and is an important first step in shape analyses (Bookstein 1991).

Because each landmark occupies a geometric location in space, multiple landmarks together can form the shape of an object. Mathematically, the shape of an object (e.g., body shape of a fish) can be explained from a series of K x M configuration matrices **X**, where K is the number of landmarks, and M is the number of dimensions (i.e., 2 or 3 dimensions) (Zelditch et al. 2004). Configuration matrix **X** can be illustrated such that:

$$\mathbf{X} = \begin{bmatrix} x_1 & y_1 \\ \vdots & \vdots \\ x_n & y_n \end{bmatrix}, \quad \text{Eq. J.1}$$

$$\mathbf{X} = \begin{bmatrix} x_1 & y_1 & z_1 \\ \vdots & \vdots & \vdots \\ x_n & y_n & z_n \end{bmatrix}, \quad \text{Eq. J.2}$$

where, each row represents a single landmark composed of (*x*, *y*) or (*x*, *y*, *z*) coordinates with a geometric location in space. Multiple objects can then be described from a series of $\mathbf{X}_{ij}$ configuration matrices (known as an array), where *i* is the *i*th specimen, and *j* is the *j*th Cartesian coordinate of the **X** configuration (Zelditch et al. 2004).

Superimposition

The entire theory of geometric morphometrics relies on the basic definition of shape (Zelditch et al. 2004). In 1977, David Kendall defined 'shape' as "the remaining geometric information after location, scale, and rotational effects are removed" (Kendall 1977). Thus by definition, shape can be mathematically defined by relatively simple algorithms, removing the differences between configurations that differ in 1) location, 2) scale and 3) orientation. These operations can easily be conceptualized by Figure J.1A, which displays two 13 landmark White Sands pupfish configurations side by side in configuration space. Each White Sands pupfish configuration contains both size and shape information. The removal of size effects, while maintaining shape information, is shown in a series of operations. These include the removal of differences in location (a process also referred to as centering or translation; Figure J.1B), the removal of differences in scale (referred to as scaling; Figure J.1C), and the removal of differences in orientation (referred to as rotation; Figure J.1D). Configurations that have undergone these transformations are free of all non-shape information. This general process aligns all objects and 'superimposes' them to a common coordinate system (Zelditch et al. 2004).

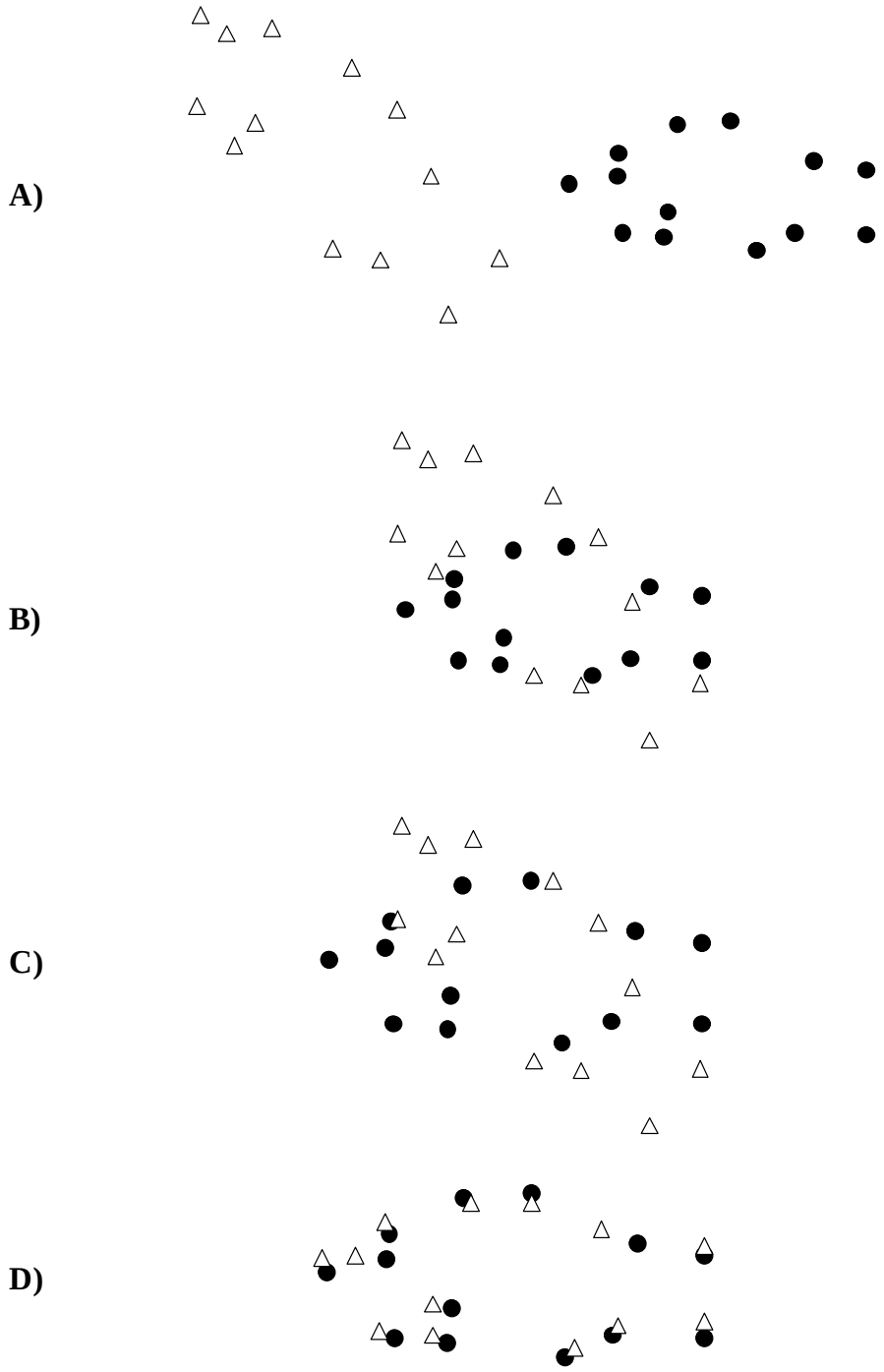

Figure J.1 Conceptualizing shape for 13 landmark pupfish configurations using superimposition methods by removal of position, scale and orientation. A) Two original 13 landmark White Sands pupfish configurations (see Chapter 2); B) location differences removed; C) scale differences removed; D) orientation differences removed. Illustrations by Brandon M. Kowalski. This figure was modified from concepts developed in Geometric Morphometrics for Biologists: A Primer, Zelditch ML, Swiderski DL, Sheets DH, and Fink WL, Pg. 12, Copyright Elsevier (2004).

The Mathematics behind Superimposition

The alignment of landmark configurations is typically achieved through least-squares superimposition methods (Rohlf and Slice 1990). The first step of superimposition, as shown in Figure J.1B, is centering. Mathematically, an object is centered by calculating the configurations centroid (defined as the center of the configuration); where, the centroid of an object is calculated by averaging the *x*- and *y*-Cartesian coordinates in the configuration matrix $\mathbf{X}_i$ (Figure J.2; Rohlf and Slice 1990). Centering is then performed by subtracting the centroid from each respective *x*- and *y*-element in $\mathbf{X}_i$ (Rohlf and Slice 1990; Zelditch et al. 2004). In effect, centering translates the original coordinates of an object to a new coordinate system were the centroid is uniquely positioned at the origin (0, 0). More formerly, centering can be performed using matrix algebra as described by Sneath (1967; *sensu* Rohlf and Slice 1990). Using matrix notation, $\mathbf{X}_i$ is centered using the following equation:

$$\mathbf{X}_{i,\text{Centered}} = (\mathbf{I} - \mathbf{P})\mathbf{X}_i, \qquad \text{Eq. J.3}$$

where,

$$\mathbf{X}_i = \begin{bmatrix} x_1 & y_1 \\ \vdots & \vdots \\ x_n & y_n \end{bmatrix} \qquad \text{Eq. J.4}$$

$$\mathbf{I}_{(\text{K xK})} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \ddots & 0 \\ 0 & 0 & 1 \end{bmatrix}, \qquad \text{Eq. J.5}$$

$$\mathbf{P}_{(\text{K x K})} = \begin{bmatrix} 1/K & \dots & 1/K \\ \vdots & \ddots & \vdots \\ 1/K & \dots & 1/K \end{bmatrix}, \qquad \text{Eq. J.6}$$

Here, $\mathbf{X}_i$ is the *i*th K x M configuration matrix of coordinates (Eq. J.1 or Eq. J.4; K being the number of landmarks and M the number of dimensions), **I** is a K x K identity matrix (Eq. J.5; i.e., a matrix with K x K (landmarks) elements were the diagonal elements are 1's

and off diagonal elements are 0's), and **P** is a K x K matrix with all elements equal to 1/K (Eq. J.6; Sneath 1967).

Scaling is the second step of the superimposition procedure described above (Figure J.1C). Scaling is performed by setting objects to unit size; where in most studies, objects are generally scaled by a measure of geometric size. The most commonly used size measure in geometric morphometrics is centroid size (Bookstein 1991; Zelditch et al. 2004). Centroid size is directly computed from the configuration matrix $\mathbf{X}_i$, as the square root of summed squared distances of landmarks from the configuration centroid (Figure J.2; Bookstein 1991).

Centroid size is independent of both the position and orientation of the configuration. Centroid size is orthogonal, and thus is uncorrelated with shape in the absence of allometry (i.e., changes in shape associated with body size; Bookstein 1986). Nonetheless, if allometric relationships are detected, ontogenetic effects are often considered using centroid size in covariate analyses (e.g., generalized linear models (GLM) or Multivariate Analysis of covariance; e.g., see Collyer et al. 2007).

There are several benefits from using centroid size. First, the leverage of any one landmark is minimized for 'large' sets of landmarks (context dependent). Second, centroid size measures the disparity of landmarks from the centroid; therefore, an estimate of size is not biased by an arbitrary choice of a linear measurement made on the organism. Third, centroid size is more analogous to measuring surface area (in 2D) or volume (in 3D) than can be obtained by using linear measurements on the organism, in most cases (M L Collyer, Pers. Comm.).

Using centroid size, scaling is easily performed. $\mathbf{X}_{i,\mathbf{scaled}}$ is calculated by dividing

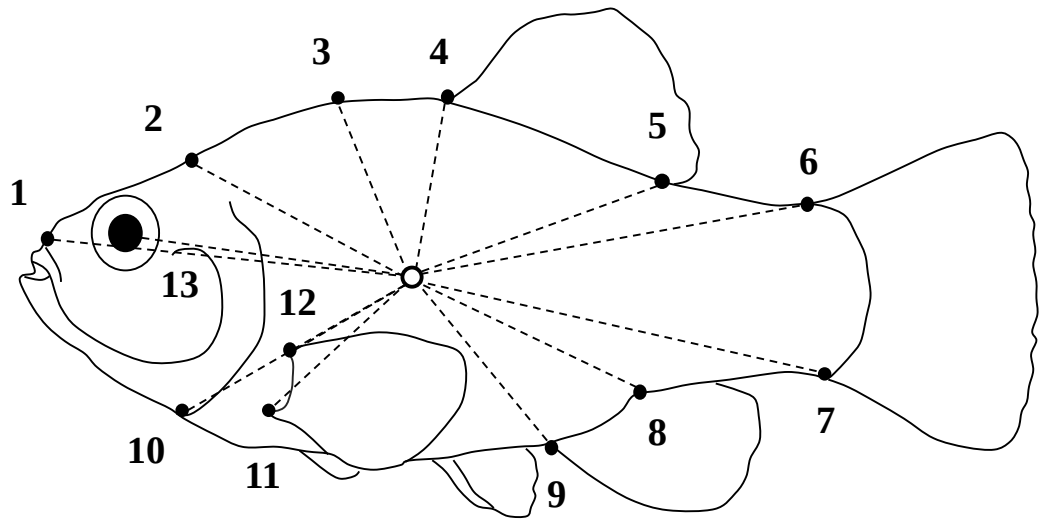

Figure J.2 Thirteen landmark White Sands pupfish configuration illustrating the centroid (unfilled) and centroid size (dashed lines). The centroid is the average of the x-, y-Cartesian coordinates (filled points). Centroid size is the square root of the summed squared distances (dashed lines) of landmarks from the configuration centroid (unfilled; actual representation). Illustration by Brandon M. Kowalski.

each *x*- and *y*- coordinate of the $\mathbf{X}_{i,\mathbf{centered}}$ configuration by its respective centroid size (i.e.,setting data to unit centroid size; Goodall 1991; Rohlf 1996; Dryden and Mardia 1998). Following Sneath (1967) (*sensu* Rohlf and Slice 1990), scaling can be performed using matrix algebra such that,

$$\mathbf{X}_{i,Centered,Scaled} = (\mathbf{I} - \mathbf{P})\mathbf{X}_i/\mathbf{S}_i, \quad \text{Eq. J.7}$$

where,

$$\boldsymbol{S}_i = \sqrt{\mathrm{tr}\left((\mathbf{I} - \mathbf{P})\mathbf{X}_i\mathbf{X}_i^t(\mathbf{I} - \mathbf{P})\right)}, \quad \text{Eq. J.8}$$

Here, $\mathbf{S_i}$ is the centroid size for $\mathbf{X}_i$, tr() is the trace of the content, **I** is the K x K identity matrix (see Eq. J.5), **P** is a K x K matrix with all elements equal to 1/K (see Eq. J.6), and $\mathbf{X}_i^t$ is the transpose of $\mathbf{X}_i$.

The final step in the superimposition process as described in Figure J.1 is rotation. Again using matrix algebra, configuration data are easily rotated following Gower (1971)

(*sensu* Rohlf and Slice 1990); where, $\mathbf{X}_{\text{i, rotated}}$ is equal to the product of $\mathbf{X}_{\text{i, centered, scaled}}$ by the rotation matrix **H**. Rotation matrix **H** can be computed directly as:

$$\mathbf{H} = \mathbf{VSU}^t, \qquad \text{Eq. J.9}$$

where, **V** and $\mathbf{U}^t$ are covariance matrices that are constrained to equal 0. Both **V** and $\mathbf{U}^t$ are found by performing a singular value decomposition (SVD), where $\mathbf{X}_1'^t\mathbf{X}_2'$ is constrained such that:

$$\mathbf{X}_1'^t\mathbf{X}_2' = \boldsymbol{U}\mathbf{\Sigma}\mathbf{V}^t, \qquad \text{Eq. J.10}$$

where, $\mathbf{\Sigma}$ is the diagonal (Eigenvalues) from the decomposed variance-covariance matrix (**S**). **S** is a diagonal matrix with $s_{ij} = \pm 1$, where the signs are taken to be the same as the corresponding diagonal elements of the $\mathbf{\Sigma}$ matrix (Gower 1971). Generally a SVD rotates, dilates, and shears matrix data (see Eq. J.11), but by constraining **U** and $\mathbf{V}^t$ to zero and taking the ±1 sign of the diagonal matrix ($\mathbf{\Sigma}$), only a rotation of shape data is performed. The equation above describes a single rotation between $\mathbf{X}_1'^t$ (reference shape) and $\mathbf{X}_2'$ (target shape), where the target shape is rotated relative to the reference shape (Rohlf 1999; Eq. J.9). In order to sequentially rotate a series of configurations, Gower (1975) further proposed an alternative approach known as a "generalized" Procrustes analysis.

Generalized Procrustes Analysis

The generalized least-squares Procrustes superimposition (GLS), commonly referred to as the generalized Procrustes analysis (GPA), is the most favored superimposition method used today, mainly due to its relative superiority to the other superimposition methods (Rohlf 2000b; 1999). Ironically, the name 'Procrustes' comes from an analogy in Greek mythology; where, Procrustes was a bandit who trapped travelers in Attica. Comparing the traveler's body size to an iron bed, Procrustes grotesquely fit his victims to size. If his victims were too tall, Procrustes would truncate them; whereas, if his

victims were too short, he would stretch them. In doing so, Procrustes minimized the difference between his victims (i.e., targeted configurations) and the bed (i.e., reference), much like that of superimposition methods used for quantifying shape (Claude 2008; Zelditch et al. 2004).

Like many superimposition methods, generalized Procrustes analyses rely on translation, scaling, and rotation to remove all non-shape information (Figure J.1). However, unlike many proposed methods, the generalized Procrustes analysis uses an iterative approach to optimally rotate multiple objects. Under such conditions, iterations are repeated until an optimal angle is achieved between the reference and all target configurations (Rohlf and Slice 1990). Iterations are necessary because of inherent shape differences among objects (i.e., not all objects have a common orientation). Differences in angles between objects are minimized using a least-squares fit approach. Optimal angles (i.e., smallest) are achieved when the least-squares fit of all target configurations from the reference shape are no longer improved. Gower suggested a tolerance of 10^{-3} or 10^{-4} (Gower 1975) before the iterative rotations between configurations cease and satisfaction has been found. For a more detailed description of how this is computed refer to Rohlf and Slice (1990).

Morphometric Spaces

The concept of morphometric spaces is abstract because most geometric morphometric data occupy higher dimensionality (Kendall 1984). Nonetheless, as objects are centered, scaled, and rotated, via superimposition methods, they move through different dimensions (i.e. morphometric spaces; Zelditch et al. 2004, Dryden and Mardia 1998, Bookstein 1986). The final position of data in space and methods of obtaining shape data

for statistical purposes can often be quite different, depending on an investigators preference. As such, the objective of this section is to 1) define the movement of data from space to space via centering, scaling, and rotating, and describe 2) why objects move from space to space, 3) the characteristics of each space, 4) the link between shape space and the loss of degrees of freedom, and 5) ultimately how shape data are collected and used for the purpose of this thesis.

As described above, each K x M matrix configuration ($\mathbf{X}_i$) retains both shape and size information of an object. This is because each landmark contains a set of Cartesian coordinates, each indicating a direction and position in space. Prior to centering, scaling, and rotating, this 'space' is commonly referred to as 'configuration space' (Dryden and Mardia 1998), because it defines all possible landmark configurations for a given K x M matrix (e.g., all 2 dimensional 13 landmark configurations represented as a 13 x 2 matrix).

Once centered and scaled, configuration data make the transition from configuration space to preshape space (Eq. J.1 - J.8; Dryden and Mardia 1998). This is because preshape space is defined by data that have been constrained via centering and scaling (as described above; Goodall 1991; Rohlf 1996; Dryden and Mardia 1998). In effect, centering and scaling constrain these data so that the squared landmark coordinates (x, y) add up to 1 (i.e., unit centroid size; Eq. J.7). This can be visualized as a set of points (representing a set of configurations) uniquely positioned on the surface of a sphere that is centered at the origin (0, 0) with a radius equal to 1 (Figure J.3; Figure J.4). Because most cannot visualize higher dimensionality, data occupying these spaces have been simplified to two and three dimensions in space. However, keep in mind that in reality, each configuration is uniquely

positioned on the surface of a K x M "hypersphere" (i.e., higher dimensionality) with a radius of 1 (Dryden and Mardia 1998).

Point A depicted in Figure J.4 represents a simplified view of a single configuration in preshape space. Notably, because point A is uniquely located at radius 1, point A can simply be defined by a direction comprised of two components: 1) latitude and 2) longitude along the surface of a hemisphere.

It is also important to note that preshape data are not yet completely defined because these data must still be rotated (Kendall 1977). Following the mathematical algorithm introduced within the superimposition section (Eq. J.1 - J.10), preshape data must also make the transition to "shape space" via rotation (Dryden and Mardia 1998). Only in shape space, with the exception of "Kendall's shape space" (not fully described here), are a group objects free of all non-shape variation (i.e., in the absence of allometry; Zelditch et al. 2004).

Characteristics of Preshape Space

Before further discussing shape space, more detail on preshape space is necessary. In reality, objects in preshape space occupy the surface of a hypersphere. Nevertheless, when depicted on a 2 dimensional hemisphere (Figure J.4 - Point A), one can see that preshape data possess non-Euclidean characteristics (Zelditch et al. 2004). This is important to acknowledge because conventional statistics do not assume non-Euclidean data (Dryden and Mardia 1998; Zelditch et al. 2004).

Objects in preshape space can be represented by a full set of rotations along a hypersphere (Zelditch et al. 2004). This can be visualized and represented less abstractly

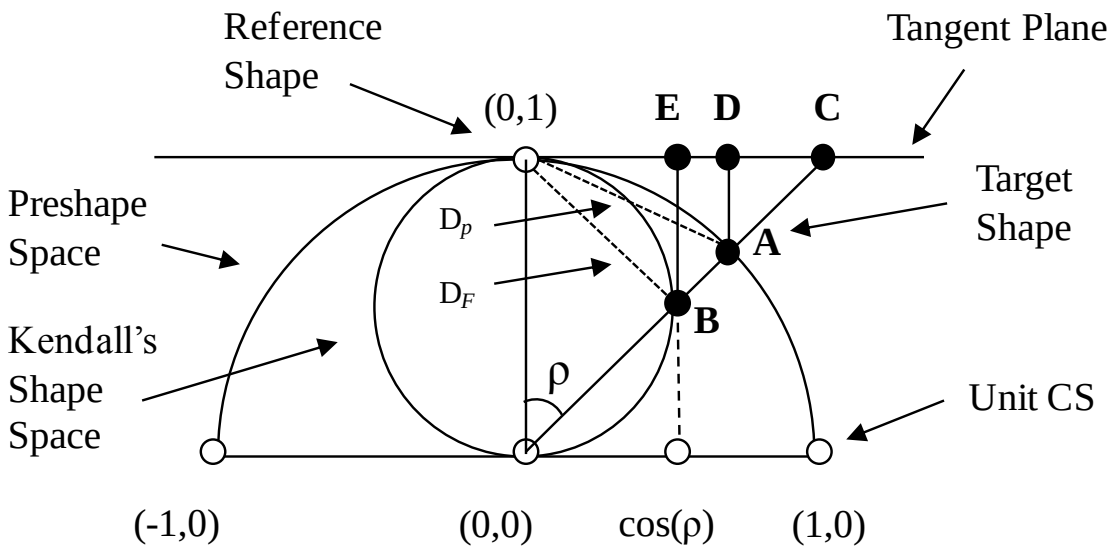

Figure J.3 A slice through part of the space of aligned configurations at unit centroid size, showing the relationships among the distance between the reference shape (at 0, 1) and object A. The semicircle is a cross-section of the space, which is a hemisphere of radius one. The length of the arc is the Procrustes distance (ρ), the length of the chord is the partial Procrustes distance (D_p), and the shortest possible distance (obtained by relaxing the constraint on centroid size (cos(ρ)), producing the configuration B is the full Procrustes distance (D_F) (see Figure J.4). Objects A and B represent data in preshape space and Kendall's shape space, respectively. Data occupying preshape space are uniquely positioned at radius 1 (0, 1); whereas data occupying Kendall's shape space are set to cos(ρ). At unit centroid size, the non-Euclidean distance along the curvature of the hemisphere is the arc length (ρ), and is equivalent to the angle between the target and reference shape in radians. Euclidean approximations can be found by: 1) Both the partial Procrustes distance and the full Procrustes distance representing stereographic projections through the interior of morphospace or 2) locations C, D, E representing orthogonal projections estimated onto a tangent plane. This figure was published in Geometric Morphometrics for Biologists: A Primer, Zelditch ML, Swiderski DL, Sheets DH, and Fink WL, Pg. 85, Copyright Elsevier (2004) and modified with permission (Thesis use only).

by defining all possible rotations for a single configuration in preshape space with a single 'fiber' (each configuration having its own set of rotations and thus its own fiber; Figure J.5; Zelditch et al. 2004). Here, a fiber is defined as a "string" of all possible rotations for a single configuration. Because each object is represented in preshape space, each fiber also has a unique position and path on the surface of the hypersphere (see fiber 1 and 2, Figure

J.5). Differences among the depicted fibers in Figure J.5 may be due to shape change, differences in rotation or a combination of both (Zelditch et al. 2004).

In order to locate the optimal rotation, the shortest distance between each fiber is found. This approach is referred to as the least-squares approach (as described above); where, optimal rotations are found by arbitrarily choosing a conveniently oriented configuration in preshape space (referred to as a reference configuration) and paired with the remaining "target" configurations (Zelditch et al. 2004). Using the algorithm described above (Eq. J.1-J.10), target configurations are optimally rotated iteratively until each target configuration to the reference configuration can no longer be improved. Applying the fiber concept, each iteration locates the minimum distance between the fiber of a target configuration and its reference (i.e., least-squares approach). The iterations cease once a

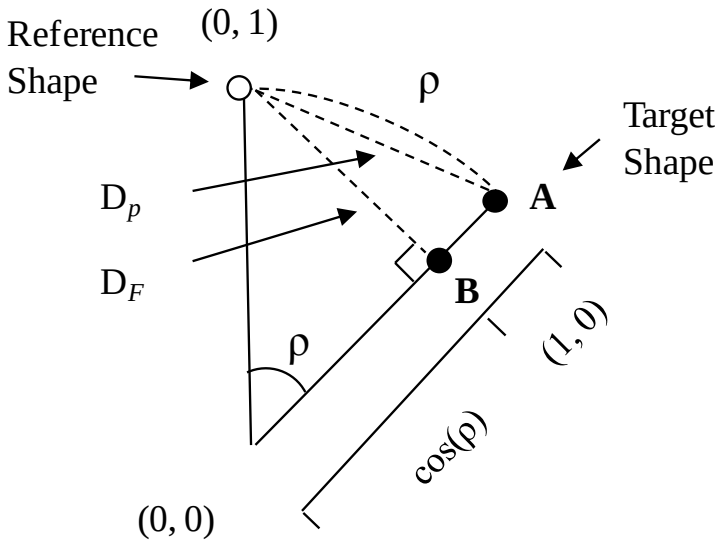

Figure J.4 A slice through preshape space showing the Procrustes distance (ρ), partial Procrustes distance (D_p), and full Procrustes distance (D_F). This figure was published in Geometric Morphometrics for Biologists: A Primer, Zelditch ML, Swiderski DL, Sheets DH, and Fink WL, Pg. 83, Copyright Elsevier (2004) and modified with permission (Thesis use only).

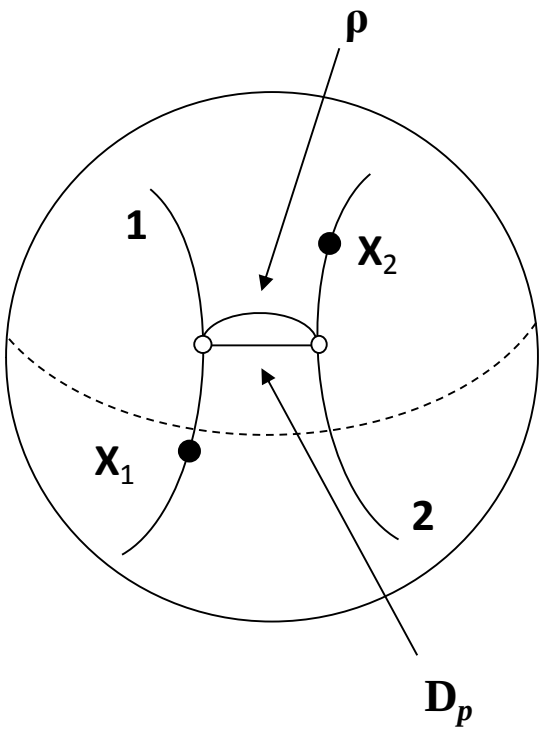

Figure J.5 Two fibers on the surface of a preshape space. Each fiber represents a full rotation of configuration X1 and X2 respectively. Filled points on each fiber represent configurations X1 and X2 at the *k*th orientation. Unfilled points on each fiber represent the optimal *k*th rotation. The inter-point distance between these orientations signify the shortest distance between both fibers. The distance measured between these two points along the surface of the hypersphere (i.e., arc) represents the Procrustes distance (ρ), whereas the Euclidean distance (i.e., chord, which passes through the interior of the hypersphere) between these two locations on the fiber is referred to as the partial Procrustes distance (D_p). This figure was published in Geometric Morphometrics for Biologists: A Primer, Zelditch ML, Swiderski DL, Sheets DH, and Fink WL, Pg. 82, Copyright Elsevier (2004) and modified with permission (Thesis use only).

minimal consensus distance between all fibers is found. At this point, the optimal rotation among all objects is found and each fiber effectively reduces to a single point (see Figure J.5).

Geometric Distances

Several geometric distances between the reference shape and target configurations exist (see Figure J.4). These include 1) the Procrustes distance (ρ; Dryden and Mardia

1998; Rohlf 1999; Zelditch et al. 2004) from one fiber to another along the surface of a hypersphere (depicted as a hemisphere in Figure J.4; e.g., point A to the reference point (0, 1) along the curve); 2) the partial Procrustes distance (D_P; Figure J.4) found between fibers through the interior of the hypersphere (e.g., point A to the reference point (0,1) through the hemisphere); and 3) the full Procrustes distance (D_F; Figure J.4) found by changing the constraints on centroid size, and allowing the target configuration to move along its radius until the shortest distance is found (e.g., point B to the reference point (0, 1) through the hemisphere or along the curve).

Several caveats exist in determining which distance to use (Zelditch et al. 2004). Choosing which distance to use is often dependent on the study and the investigators preference. For example, distortions in Euclidean tangent space may result when distances between the reference and target configuration become too large. Such conditions may arise when assuming homology among multiple species in a study (Stayton 2006). In this context, inferences based on distances in the Euclidean tangent (described below) will be unreliable; whereas, non-Euclidean distances are curved and are generally difficult to work with statistically. Despite such risks, Euclidean approximations are generally estimated (Dryden and Mardia 1998).

Euclidean Approximations

Euclidean approximations can be found tangent to the constructed spaces described (see Figure J.4 for various Euclidean approximations; Rohlf 1996, 2000a). Here, projections made from unit centroid size (i.e., Points E and D to (0, 1), Figure J.4; Goodall 1991, Rohlf 1996; Dryden and Mardia 1998) are commonly referred to as orthogonal approximations; whereas, projections through the interior of the hypersphere (i.e., Point C

and distances D_p and D_F, Figure J.4) are referred to as stereographic approximations. A more detailed understanding of morphometric spaces can be found in Dryden and Mardia (1998), and Zelditch et al. (2004).

Original shape variables occur in a curved shape space, and thus must be projected onto a tangent plane before performing normal Euclidean statistical operations (Rohlf 1996, 1999; Zelditch et al. 2004). For the purpose of the data collected in this disquisition, Euclidean approximations were made from orthogonal projections at unit centroid size (i.e., the orthogonal projection of point A to point E, Figure J.4; Goodall 1991; Rohlf 1996; Dryden and Mardia 1998). Projections such as these may introduce distortions in the data similar to differences that appear when a globe is projected onto a 2-dimensional map.

To test if these projections were sufficiently small, Procrustes distances (ρ; Figure J.4) were regressed against the orthogonal projected distances along the tangent plane. Computations were performed using the program tpsSmall v. 1.20, and a strong coefficient correlation of $r = 0.99$ between the Procrustes and projected distance was set to assure that projected data were small enough for meaningful inferences (Rohlf 2003; Stayton 2006).

Variation among the 13 landmarks used appeared to be equally distributed from the mean (see Figure 2.2). When tested, Procrustes and Euclidean distances between all specimen showed a strong linear relationship ($r = 1$), demonstrating that orthogonal approximations could be used without concerns of distortion.

Degrees of freedom

As described above, two-dimensional data starts out with 2K variables (i.e., *x*-, *y*-Cartesian coordinates). In the course of superimposing shape data a total of 4 degrees of freedom are lost (i.e., 2K-4 shape variables; Rohlf 1996). The centering of an object in

configuration space requires two degrees of freedom (d.f.); one d.f. for each respective *x*- and *y*- coordinate. Scaling constrains the data to unit centroid size, removing yet another d.f., and rotating constrains the data to a common orientation, losing the final d.f. for a total of 4 d.f. lost or 2K-4 free shape variables.

Because data are transferred from space to space after each constraint (i.e., centering/scaling and rotating), the number of dimensions of preshape space and shape space directly corresponds to the number of constrained parameters used to transform the data (Zelditch et al. 2004). In addition, each coordinate shares a fraction of the degrees of freedom. Thus, although 2K-4 variables exist, all 2K coordinates (i.e., landmarks) are maintained. Consequently, there are too many shape coordinates for the number of degrees of freedom. As you will see in the following section, this is a minor problem which can be easily circumvented by ignoring the dimensional loss and testing for shape differences using resampling based statistical methods.

Statistical Procedures

Ordination Methods

Usable shape data are generally obtained by performing relative warp analyses (RWA) on partial warps of aligned shape data through a thin-plate spline analysis (TPS) (Bookstein 1991). The relative warps scores are then frequently used to describe shape variation among groups and the association of shape and other variables (e.g., size) using multivariate linear models (Bookstein 1991; Zelditch et al. 2004).

A relative warp scores analysis is a statistical analysis of the dispersion of shape (Rohlf and Marcus 1993). One purpose of the thin-plate spline analysis is to produce a series of partial warps, which are later rotated via a principal component analysis to

produce relative warps. In effect, RWA is simply a weighted principal component analysis (PCA) of shape variation. Therefore, for the purpose of this disquisition, PCA and RWA herein are discussed together. Furthermore, because thin-plate spline analyses also generate deformation grids, they are also discussed later.

A principal component analysis (PCA) is used to describe shape variation among individuals by finding the most information in the fewest dimensions by means of rigid rotations of linear combinations within data space (Rencher 2000). PCA is beneficial because all variation described can be reduced to only a few components, while preserving geometric relationships of anatomical features throughout analysis (Adams and Rohlf 2000, Rohlf and Marcus 1993; Bookstein 1989; see thin-plate spline paragraph below).

PCA is performed by minimizing the sum of the squared distances of points away from each orthogonal line of fit (Zelditch et al. 2004). Principal component (PC) scores are then determined by the orthogonal projections of specimen onto principal component axes (Eigen vectors- A_i), with each score representing the distance of a specimen from the mean in each principal component direction (Zelditch et al. 2004). In other words, the principal components are the axes of a new coordinate system; where, individual variation is rotated and translated into a more convenient orientation where relative positions are left unchanged. Because these new variables are created from the original shape variables, PCA is known as an "ordination method" (Zelditch et al. 2004).

Mathematically, PCs are constrained under an orthonormal basis. The first PCA constraint requires that the total variance remains unchanged between the new and original data. Such constraints are performed by setting each principal component (PC) or

Eigenvector (A_i) to 1 (unit vector). Second, all progressive linear combinations (PCs) must be orthogonal to each other. To fulfill this requirement, the covariances among variables are set to zero. Third, the last constraint requires that the variance of the first principal component (PC I) is maximized, and that subsequent PC axes are ordered to explain sequentially less variation under the first two constraints listed above. This is performed by ranking the PCs (Eigenvectors) by the amount of variance captured (Eigenvalue - λ). Notably, the proportion of variance explained by any Eigenvector is equal to λ_i divided by the sum of all Eigenvalues (see below).

Under these conditions, orthogonal Eigenvectors are obtained by performing a SVD (i.e., Eigen analysis; Rencher, 2002) on the p x p variance-covariance (**S**) matrix of the original data set, such that:

$$\mathbf{S} = \mathbf{V}\mathbf{\Lambda}\mathbf{V}^{t}, \qquad \text{Eq. J.11}$$

where SVD decomposes (see Eq. J.9 for resemblance) the variance-covariance matrix **S** into three components: 1) **V**, which is a p x p rotation matrix, 2) $\mathbf{\Lambda}$ which is a p x p diagonal matrix composed of Eigenvalues (λ_i; also known as singular values), and 3) $\mathbf{V}^t$ which is second p x p rotation matrix. The original data are then transformed to new coordinates (i.e., PC scores) by projecting the original data on **V** (i.e. $\mathbf{Y}_{\boldsymbol{Rotated}} = \mathbf{YV}$). Individual scores from these methods can then be plotted and visually interpreted or used for subsequent analysis.

Thin-Plate Spline Analysis

Shape differences in shape space can be visualized through thin-plate spline (TPS) deformations in light of D'Arcy Thompson's original deformation grid in 1917 (Thompson 1917). Thin-plate spline procedures depict the "bending" of a grid placed on one object as

it is mapped onto another object, allowing one to interpolate inter-landmark differences, while taking all landmark displacements into account (i.e., all landmark differences are 'smeared' across the grid; Bookstein, 1989). The resulting deformation plots resemble whole organisms, providing a visually interpretable appreciation of shape differences (e.g., see Figure 2.2).

Metaphorically, TPS deformation grids are analogous to the bending of a thin steel plate (*sensu* Bookstein 1989). Imagine two configurations, 1) an overall consensus configuration and 2) a targeted population consensus, that are each represented on a thin metal plate (consensus meaning mean body shape). Now imagine superimposing these two plates (i.e., configurations) such that the target is overlaying the consensus configuration. Next, weld a bar to each corresponding landmark (e.g., landmark 13 of the targeted configuration to landmark 13 of the consensus configuration), where larger inter-landmark differences accrue longer bars and smaller distances, shorter bars. Because each corresponding inter-landmark distance generates a greater degree of unevenness across the targeted plate, one plate cannot be flat. Therefore, bending occurs into a third dimension, resulting in the warping of a steel plate. Metaphorically these differences among corresponding landmarks demonstrate a bending energy across a plate, where longer bars generate a greater amount of energy and thus a greater amount of bending or shape change (see Figure J.6).

Mathematically, a deformation is a smooth differentiable (i.e., continuous) function that maps one configuration to the corresponding points of another. As a result, a TPS analysis can be used as an interpolation function to visualize differences among group means (Bookstein 1989).

Deformations can be decomposed into both affine (uniform) and non-affine (non-uniform) components (Figure J.7; Zelditch et al. 2004). There are six independent types of affine deformations for two-dimensional landmarks. These include: 1) translation along the vertical axis, 2) translation along the horizontal axis, 3) scaling, 4) rotation, 5) compression/dilation, and 6) shearing (i.e., the deformation a configuration in which parallel internal surfaces slide past one another due to scaling of a row or column; Zelditch et al. 2004).

All affine deformations include parallel transformations, which require no bending energy (Bookstein 1989). In contrast, non-affine components include all transformations that consist of non-parallel deformations, which involve the metaphorical bending

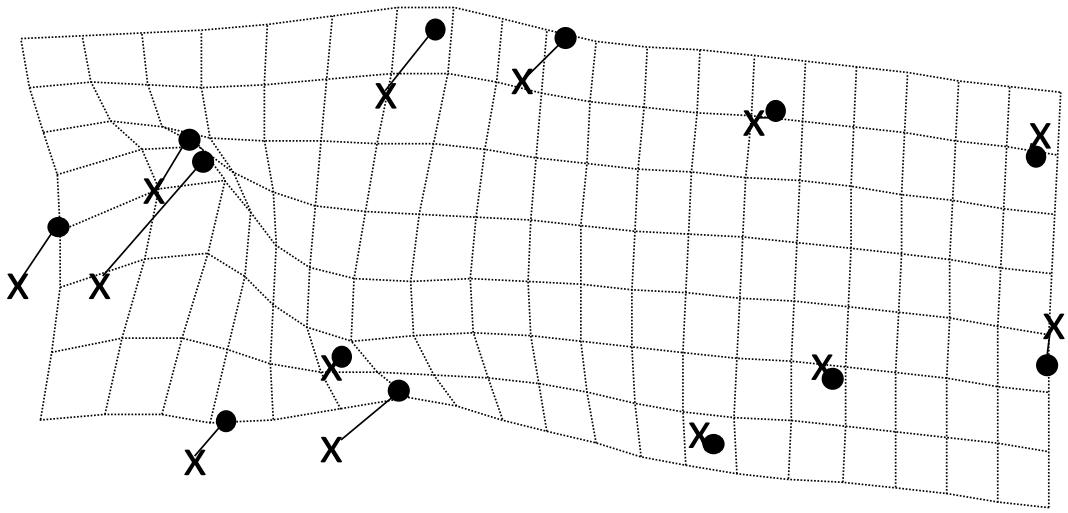

Figure J.6 Illustration of the steel plate metaphor (Bookstein 1989) using 13 landmark White Sands pupfish configuration. Consensus (crosshairs) and target (black points) configurations represent landmarks positioned on two independent overlapping metal plates. Solid lines represent welded bars and demonstrate bending energy between plates; where, longer bars generate a greater amount of energy and thus a greater amount of bending or shape change. Deformation grid (dotted) depicts shape change between the two configurations. Figure for demonstrational purposes only. Illustration by Brandon M. Kowalski.

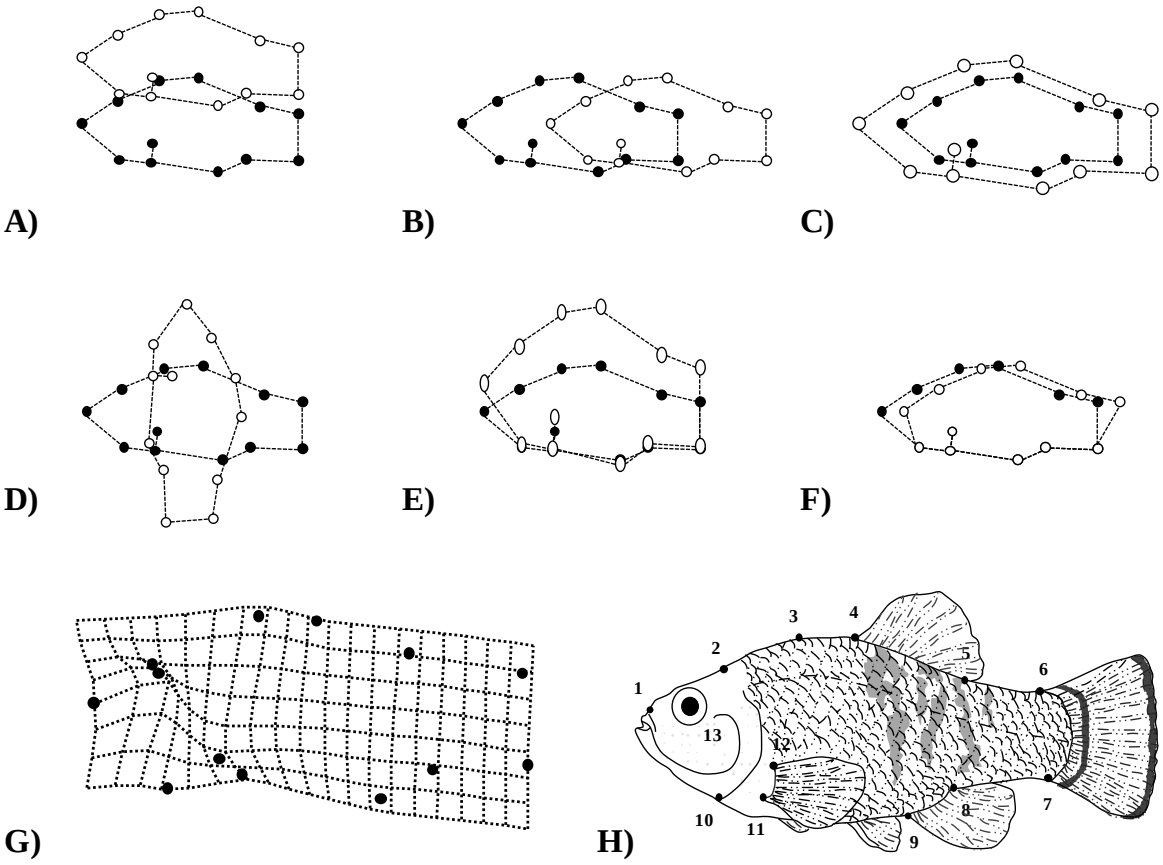

Figure J.7 A summary of affine and non-affine components generated within a thin-plate spline analysis. Six affine transformations exist, including A) translation along the vertical axis, B) translation along the horizontal axis, C) scaling, D) rotation, E) compression/dilation, and F) shearing. Non-affine components, called partial warps, include highly localized changes. G) Non-affine deformation depicting a contraction between the center eye and cranial/body junction and an expansion of the mandible relative to the head region. H) White Sands pupfish depicting the placement of 13 anatomical landmarks. Illustrations by Brandon M. Kowalski.

described above. Non-affine deformations include localized changes, which can further be decomposed using the TPS interpolation function (described above) to produce components called partial warps (Figure J.7G; Bookstein 1989; Zelditch et al. 2004). Each partial warp has one 2 dimensional partial warp score vector, which provides orientation information (i.e., partial warp scores have an x-, y- component, indicating the direction of the partial warp; Zelditch et al. 2004). Thus, it is the partial warp scores that express the localized or non-affine contribution.

The combination of affine and non-affine components describes the total observed shape change. Interpretations should be made from the overall deformation (i.e., using both affine and non-affine components combined; Zelditch et al. 2004). In order to visualize subtle differences, observed differences are often accentuated by a factor of 2 or greater (M L Collyer, Pers. Comm.). Need-less-to-say, the greater the magnification the more unrealistic the deformation becomes.

All mathematical and statistical computations described above can be conducted using specialized geometric morphometric software and conventional statistical programs (see below for further detail). For a more detailed explanation of relative warp scores or thin-plate splines, refer to Bookstein (1989, 1991).

APPENDIX K. GLOSSARY OF SELECTED GEOMORPHOMETRIC TERMS AND SYMBOLS

The following words or symbols are regularly used throughout this disquisition and heavily used in the geometric morphometric and statistic field. The definitions and symbols used throughout this disquisition have been developed from the readings in Zelditch et al. (2004) and other peer reviewed sources, and are not *verbatim* unless otherwise specified by quotations. Some mathematical abbreviations or symbols were created (denoted by an asterisk) to avoid ambiguity or confliction among algorithms.

Terms

Arc	The curved distance along the surface of a hypersphere from the reference and target object positioned in preshape space. Also known as Rho (ρ).
Affine Deformation	A transformation that leaves parallel lines parallel. There are six affine transformations. These include: 1) translation along the vertical axis, 2) translation along the horizontal axis, 3) scaling, 4) rotation, 5) compression/dilation, and 6) shearing.
Cartesian Coordinates	Set of x- and y- coordinates within a coordinate system.
Centering	**(**Refer to translation).
Centroid	Center of a configuration. Centroid is designated by a set of Cartesian coordinates and computed by taking the average of each respective landmark coordinate within the configuration.
Centroid Size	The square root of summed squared distances of landmarks from the configuration centroid. Abbreviated as CS.
Chord	Inter-hypersphere distance between a target and reference object located in preshape space. Also referred to as the partial Procrustes distance (see below).

Configuration	The collective set of (K) landmarks representing the object of interest.
Eigenvalue	The explained variance among eigenvectors (PC axes). Symbol is λ. See Eigenvector.
Eigenvector	Non-zero orthogonal vectors (A_i) consisting of transformed values that make up the principal component axes.
Euclidean	Pertaining to standard geometry.
Fiber	"String" of all possible rotations of a single configuration in preshape space.
GPA	**Generalized Procrustes analysis;** An algorithm that translates, scales, and iteratively rotates each configuration or object to an arbitrary reference shape.
Hyperspace	Multiple dimensional space.
Hypersphere	Multidimensional sphere. Objects in hyperspace are located on a sphere in K x M dimensions.
Iteration	Repeated sequence of mathematical operations.
Kendall's Shape Space	Morphospace named after D. G. Kendall. Kendall's shape space is defined by target objects that have been translated, scaled, rotated, and set to cos(ρ) centroid size. Note only target objects occupy Kendall's shape space.
Landmark	A homologous-anatomical position on an object. Landmarks are stored as Cartesian coordinates (i.e., x- and y-coordinate). Abbreviated as (LM). See landmark typology.
Landmark typology	Bookstein (1991) described fixed landmarks into 3 categories, type I, II, and III. Type I landmarks refer to landmarks at discrete juxtapositions; Type II as minima or maxima; and Type III as extremal points.
Least-squares Method	Mathematical criteria for locating the minimum summed squared distances among objects. Used in GPA to optimally rotate configurations.
Non-affine deformation	The component of a deformation that is not uniform or parallel. Antonym of affine deformation.

Non-Uniform Transform.	Not a uniform or parallel transformation. Also referred to as non-affine. See non-affine deformation.
Object	Alternative for configuration.
Orientation	Rotational position of an object in morphospace.
Orthogonal Projection	An orthogonal projection of preshape data or Kendall's shape data located on the surface of a hypersphere onto a plane (see tangent plane).
Orthonormal	A condition in linear algebra were a pair of vectors are both orthogonal and set at unit length.
Partial Warp	The components of non-affine or non-uniform change produced by a thin-plate spline analysis.
Partial Warp Scores	"Coefficients indicating the position of an individual, relative to the reference, along partial warps (Zelditch et al. 2004)."
PCA	**Principal Component Analysis;** an ordination method that rigidly rotates original data onto a new set of variables. PCA reduces the dimensionality of multivariate data by capturing variation onto principal components (PC); where each PC sequentially captures less variation (also refer to RWA).
Preshape Space	Morphospace occupied by objects that have been constrained by translation and scaling. Objects in preshape space have 2K-3 dimensionality.
Reference	A configuration arbitrarily chosen for optimally rotating targeted configurations or objects.
RWA	**Relative Warp Analysis;** similar to PCA, but "relative warps are of partial warp scores that are weighted to emphasize components of high or low energy bending (Zelditch et al. 2004)."
Rotation	Orientation of an object. Objects are rotated respective of an arbitrary reference object.
Scaling	Magnification or the universal manipulation of an object. Coordinate relationships are preserved.
Shape	[1]Defined by Kendall in 1977 as "the remaining geometric information after location, scale, and rotational effects are

	removed". [2]May be used alternatively for object or configuration (e.g., reference shape).
Shape Space	Morphospace occupied by objects that have been constrained by translation, scaling, and rotation.
Stereographic Projection	A projection of preshape data located on the surface of a hypersphere onto a plane (see orthogonal projection and tangent plane).
Tangent Plane	The location tangent to the surface of the hypersphere at coordinates (1, 0) or the reference position.
Target	A configuration or object that is being rotated relative to a reference.
Translation	Relocating an object, composed of x- and y- Cartesian coordinates, onto a new coordinates system. Also referred to as centering.
Type I SS	**Sum-of-Squares** (Sequential); The sequential improvement of the error SS when one or more predictor variables are added to the regression model (order matters). The SS for all effects add up to the total SS.
Uniform Transformation	See affine deformation.

Symbols

A*i*	Eigen vector representing the *i*th principal component axes.
Alpha (α)	In statistics α is the type I error (i.e., rejecting the null hypothesis when it is actually true). Alpha throughout this disquisition has been set to 0.05.
D_p	Partial Procrustes distance. The inter-hypersphere distance between the target and reference object located in preshape space. Also referred to as a chord (see definition above).
D_F	Full Procrustes distance. The inter-hypersphere distance from target objects occupying Kendall's shape space.
K	Number of Landmarks.
Lambda (λ)	Eigenvalue representing the amount of variation captured in each progressive principal component.

M	Number of dimensions.
Rho (ρ)	The angle between the target and reference object in preshape space. Alternatively referred to as the arc length between the target and reference object on the surface of a hypersphere.
$\mathbf{S}_i$	The centroid size matrix of $\mathbf{X}_i$.
$\mathbf{X}_i$	Notation for K x M (i.e., landmark by dimension) sized matrix. Here *i* represents the *i*th Matrix. Matrices are indicated in bold font.

APPENDIX L. REFERENCES

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